

The case against a perpetual NPT

The conference of the members of the Nuclear Non-Proliferation Treaty must strive to continue the treaty, but not to make it perpetual; the world will need regular reminders of the hazards of proliferation for decades to come.

THE conference of the members of the Nuclear Non-Proliferation Treaty (NPT) due to open in April at Geneva will be a formative experience for all concerned. There are two crucial questions to be decided. Will the treaty continue in being? And will its members agree that it should continue in being indefinitely? The hope must be that the former question will be answered in the affirmative. The second is trickier. Even those who regard the NPT as a linchpin of international security should not be too downcast if the members settle for another fixed term.

As treaties go, the NPT is every bit as important as its well-wishers say. In 25 years, it has been a powerful restraint on the spread of nuclear weapons. Those who doubt that have only to consider what the world would now be like if there had been no presumption of law-breaking to level at countries such as Iraq and North Korea (both members of the treaty) in the past few years. Similarly, without the NPT, the chaotic breakup of the Soviet Union since 1990 might well have made to seem legitimate the sale of chunks of one of the world's largest stockpiles of fissile material. Of course, the NPT has not been ideal. Both Iraq and North Korea chose to break the rules, but were found out. More seriously, several important putative nuclear powers (Israel, India and Pakistan, for example) remain outside the treaty. Yet the NPT has helped to make the world a safer place.

Can that continue? Next month's conference will raise a piquant dilemma for the non-nuclear members of the treaty. From the outset, many of them have complained that the treaty is asymmetrical between nuclear powers and themselves; non-nuclear powers must put up with inspection of their nuclear installations, but nuclear powers are formally exempt. And a quid pro quo for that asymmetry, the undertaking in the NPT that the nuclear powers would provide technical assistance in the exploitation of civil nuclear technology to those in need of it, now seems hollow.

Yet in normal times, the traditional nuclear powers would have been able to boast that they have amply fulfilled their other promise in the NPT to pursue measures of strategic arms control "in good faith". Central Europe is no longer bristling with nuclear weapons, thanks to the agreement on strategic weapons of intermediate range. There are also agreements signed, but not yet ratified, on the balanced reduction of weapons of longer range, while both the United States and Russia (inheritor of the Soviet Union's obligations) appear to have given up ambitions to build defences against strategic weapons. Is that not enough, the nuclear

powers would ordinarily be asking?

They know, without waiting for the answer, that present circumstances are too confused for even this splendid record to suffice in present circumstances. One snag is the ambiguity of China's position on proliferation and on strategic issues in general; there has been nothing like the dialogue with the West that paved the way for the eventual *rapprochement* with the former Soviet Union. Similarly, the continuation of Israel's cryptonuclear status, as that of India, goads other states towards proliferation. That is why the nuclear powers now seem ready to go a big step further, offering a comprehensive test-ban treaty (CBT) as further earnest of their good faith. Will that pull round the non-nuclear doubters? Especially now that the United States has abandoned a condition allowing the resumption of testing after a ten-year break, there is a good chance.

Even so, the non-nuclear powers will not willingly sign up for a perpetual continuation of the NPT — nor should they. Fifty years after the nuclear explosion at Alamogordo in the Nevada Desert, one thing is certain: the knowledge of nuclear weapons, of their destructiveness and of their military influence as deterrents will not simply melt away. As the decades ahead roll by, it is inevitable that bigger or smaller powers will hanker after these trappings of power. Who can say what the effects of such developments would be on now-pacifistically minded governments? At least while the present nuclear powers intend keeping part of their stockpiles (and intend keeping them in working order), even a CBT will not persuade non-nuclear powers to sign a perpetual treaty.

Indeed, a perpetual treaty, by making it seem that the problem of proliferation had been solved, would weaken the fight against proliferation. The quinquennial review conferences of the NPT, often irritating for the nuclear powers, have nevertheless kept the need to battle against proliferation on the public agenda. It is not as if a CBT would be the last step towards nuclear sanity. Why not, next, an agreement (safeguarded by inspection) to halt the production of fissile material for other than peaceful purposes? And who is to set up and manage the stockpiles of fissile material that will require international safeguarding when civil nuclear power comes into its own again? Only regular formal discussions between like-minded governments will keep those questions alive. That is why the best outcome of this year's conference will be a renewal of the NPT for 25 years, with regular meetings every five years. □



Mergers mean risks

The planned merger of Glaxo and Wellcome raises questions broader than the interests of shareholders.

THE House of Commons Select Committee on Science and Technology chose a good target last week when it cross-examined Sir Richard Sykes, the chief executive of Glaxo plc, on the impact on British pharmaceutical research of his company's bid for its smaller UK rival Wellcome plc. But it then selected the wrong ammunition.

The committee was chiefly concerned with the effect of the proposed merger on jobs in research and development. It started from the view that such losses would weaken Britain's science base. What it should have asked is whether Glaxo, by staking its future on remaining a "vertically integrated" company, stretching from basic research to drug delivery, is taking an excessively high risk. The strategy contrasts with that of other companies, such as SmithKline Beecham and Merck. Its failure could have major consequences for the British pharmaceutical industry.

The answer to the question that might have been asked is "probably not". But the outcome is by no means certain. Glaxo will now depend more than ever on the ability of its research arm to find new therapeutic agents. Sykes insists that Glaxo is committed to maintaining a strong investment in both fundamental research, particularly in molecular genetics, and in the new technology for screening new agents for biological effectiveness. (The company's recent purchase of the US biotechnology company Affymax is one step in that direction.) The gamble is whether the days of serendipity are over.

If the committee had pursued that line, it would have illuminated the central paradox in the proposed merger: the claim that reducing the overall spending by Glaxo and Wellcome on research and development will actually increase the ability of the merged company to produce new products. From the outside, the logic is not self-evident, however much Glaxo argues the value of 'rationalization' (that is, cost-cutting) to justify the price it is offering for Wellcome shares. But there may be much less overlap between the two companies than some have suggested. According to Wellcome officials, there are only four research projects (out of about 80) in which the two companies compete directly. The major duplication is in the development phase, for example in toxicity testing. And that is where the largest staff cuts are likely to be made — and where the greatest impact of new screening technologies, such as those offered by Affymax, are likely to be felt.

The labour unions representing those working in these fields are right to be concerned about their members' job security. The argument that cuts are inevitable as the global pharmaceutical industry adjusts to unaccustomed economy will not ease the pain for those who find themselves out of work. But the argument is probably also correct. This is not a time for the faint-hearted in pharmaceuticals. Yet those who remain (including the shareholders) will properly

remain nervous. Glaxo's position in the marketplace is largely due to a single product, the antiulcer drug Zantac. By staking its future on similar blockbusters, Glaxo is putting all its eggs in one basket. There may be no choice, but the select committee may regret not having asked. □

Derivatives sink banks

The collapse of a British merchant bank is not a signal to ban risky trading, but an argument for transparency.

THE collapse last weekend of Barings, London's oldest merchant bank, will again raise the complaint that the money markets have become too complicated for ordinary mortals, and even banks, to understand. But that is the wrong lesson. What seems to have happened is that a young man at the bank's Singapore branch had been trading in financial instruments called 'derivatives', which are merely promises to buy or to sell at a predetermined price and at some future date an underlying commodity. In Barings' case, the commodity was the value of certain stocks on the Tokyo stock exchange. The young man seems to have committed Barings to at least £600 million of outlay in the next few weeks — a sum that exceeds the bank's capital value.

There is nothing new about derivatives. For decades, traders in Chicago have amused us with their fondness for buying parts of animals — 'pork bellies', for example — on such a basis. It is a sensible economic practice. A person knowing he needs a certain number of them in a few months will prudently pay a potential supplier a small sum of money in advance so as to secure the terms of the eventual deal. Such an arrangement will also often suit the supplier, who may welcome knowing how much he will get for what he supplies. But equity requires that a person with an option to buy or an option to sell should be free to sell the obligation thus acquired to somebody else.

The much more common use of options now is in the management of foreign currency. A British company expecting an income in the United States by the end of 1995, and needing then to turn US dollars into sterling, may decide now to buy an option for the delivery of so much sterling at a predetermined exchange rate. If the US dollar then weakens against sterling, the anticipated income will be maintained; otherwise, the company that makes the contract will 'lose', but usually that risk takes second place besides the security the contract brings.

The real risks arise when people and organizations use the options markets as if the options were themselves commodities. The cost of an option may be a small fraction of the value of the underlying commodities. The money that changes hands is therefore a small part of the risk if things go wrong. Barings is only one of several organizations to have come unstuck in the past few months. Trading of this kind cannot be banned, for that would be inequitable. But those who gamble in this way should not do so with other people's money unless they warn them in advance, which is what shareholders (and regulators) are for. □

Despite early fears, first Republican budget cuts bode well for science

Washington. The budget for basic scientific research has emerged virtually unscathed from the first round of Republican budget cuts in the US House of Representatives, strengthening hopes that the major science agencies will escape the zeal of the new majority in congress.

The House is in the process of passing two rounds of budget rescissions — sets of proposals to withhold money already allocated for the current financial year which ends on 30 September. These represent almost \$20 billion in savings. But while the cuts will affect plans to build research laboratories and industrial technology programmes, support for basic research is virtually untouched.

The cuts are the first step in the 104th Congress's promised assault on public spending. But they reveal a set of priorities producing some relief in the science community. "Given the magnitude of the cuts in other programmes, one can regard this as a good sign," says Al Teich, director of science policy at the American Association for the Advancement of Science (AAAS).

In a package that cut a total of \$4.3 billion, most of it from education and social programmes, the House appropriations subcommittee responsible for the Departments of Labor, Health and Human Services, and Education, chaired by John Edward Porter (Republican, Illinois), cut just \$70 million from this year's \$11-billion budget for the National Institutes of Health (NIH).

In addition, the NIH will lose \$50 million in construction funds, largely for a new administrative building it no longer wants, and \$20 million for an extramural programme designed to help renovate university laboratories.

Similarly, a package of cuts totalling \$9.4 billion from the subcommittee responsible for Veterans' Affairs, Housing and Urban Development and 'independent agencies', chaired by Jerry Lewis (Republican, California), axed only \$66 million from the budget of the National Aeronautics and Space Administration (NASA) — and nothing from that of the National Science Foundation (NSF).

The NASA cuts would save \$27 million by closing the Consortium for International Earth Science Information Network (CIESIN) at Saginaw, Michigan. In addition, \$10 million would be cut from the operation costs for the Hubble Space Telescope, \$25 million from the \$1.3-billion Mission to Planet Earth Programme, and \$1 million from the cost of flying the private jet used by Dan Goldin, NASA's administrator.

In a separate package already approved by the full House, NASA lost \$400 million for a new wind tunnel complex, and NSF lost \$132 million for facilities construction (see *Nature*, 373, 552; 1995). In both cases, the agencies involved had said they were not ready to spend the money appropriated by last year's Congress.

At the Department of Energy, members

of the appropriations subcommittee have proposed cuts of \$35 million in solar energy research, \$50 million in energy conservation programmes and \$145 million in environmental remediation. But research in nuclear fission and nuclear fusion, as well as basic research, was left untouched.

As expected, the research agency hit hardest by the cuts is the National Institute of Standards and Technology (NIST), whose technology programmes have been rapidly expanded by the Clinton administration. The package already passed by the house cut \$107 million this year from NIST's Advanced Technology Program, and an appropriations subcommittee proposed further cuts of \$20 million from NIST intramural research and \$26 million from the Manufacturing Extension Partnership, a programme to build manufacturing technology centres around the country.

Meanwhile, other appropriations subcommittees have cut \$17 million — 10 per cent of the budget — from the National Biological Service, and a modest \$650,000 from Congress's own Office of Technology Assessment. So far the US Geological Survey, widely seen as a target of conservative Republicans, has not been cut at all.

Both NIST and the Department of Energy have issued statements denouncing the cuts, but the other science agencies have been maintaining an appropriate silence. But the cuts themselves may be vetoed by the Clinton administration.

The real power to impose its priorities will come when the House of Representatives sets the next budget, which covers the fiscal year beginning in October 1996. Observers say the cuts show a pattern familiar from the early Reagan years, when basic science was protected while education and technology programmes were cut.

Given the wave of cuts elsewhere, says Teich, the proposals are "perhaps an indication that Congress is going to go easy on research spending." He points out that this would be consistent with what Republican leaders such as Newt Gingrich (Republican, Georgia), the House Speaker, and Robert Walker (Republican, Pennsylvania), the chairman of its science committee, have been saying since November.

Colin Macilwain

French blood affair

Paris. The list of those charged with 'poisoning' in France's contaminated-blood affair rose to eight last week, with the indictment of Gérard Jacquin, the former director of bioindustry at the National Blood Transfusion Centre (CNTS).

D.B.

Spirits enter into an electronic medium

London. Visitors to a 'showcase of information technology' in Brussels last week had the opportunity to view on a computer screen an historical image of Sir Arthur Conan Doyle (right), courtesy of the British Library. The 'psychic photo', from the early twentieth century Barlow Collection, is part of a sample of high-resolution digital images mounted on a prototype viewing system set up by the library.

The system was chosen as one of 150 exhibits, organized to coincide with a G-7 ministerial conference on the Information Society in Brussels on 24–26 February. The exhibition is designed to increase awareness of the developing information society and to show some of its potential benefits.

The British Library launched the system a year ago to speed access to images in the library's holdings of books, manuscripts and photographs.



Users can search each collection using subject words, shelf marks or other indexes and once they have retrieved the images they want, they can view their selection as a high-resolution image on screen.

M.V.

AIDS activists seek speedy access to protease drugs...

Washington. Attempting to tackle one of the thorniest issues posed by the newest class of antiviral AIDS drugs, namely protease inhibitors, the US National Task Force on AIDS Drug Development last week set up a group to decide who should qualify for compassionate use of the drugs while they are undergoing the approvals process.

Three companies — Roche, Merck and Abbott — have protease inhibitors undergoing human trials. Encouraged by promising experimental results and the fact that these drugs seem to have fewer unpleasant side effects, AIDS patients are pressing the companies to apply for accelerated approval as soon as possible. This is available for drugs that treat life-threatening diseases, and the three companies could be ready to apply towards the end of the year.

While the approvals process goes ahead, AIDS activists argued last week (as they have done in previous cases) for concurrent programmes of expanded access for compassionate use. Expanded access programmes exist in a variety of bureaucratic guises, but all have the same aim: to provide treatment for people who have no other medical option in the period just before a new therapy gains approval. The term "salvage therapy" used by AIDS activists says it although Philip Lee, an Assistant Secretary for Health and head of the task force said that he would prefer them to talk of life-sustaining therapies.

Expanded access is always a difficult issue, but it is amplified in the case of protease inhibitors. This class of compounds has a complex structure that the companies say is particularly difficult to manufacture. All three are increasing production, but yields are low. Abbott, for example, is getting yields as low as two per cent.

To compound the difficulty, each patient needs about 1 kg of the drug a year, which is "50 to 100 times as much as other antiviral drugs", says Edward Scolnick, president of the Merck Research Laboratories and a member of the task force. So the question to be answered is how should the limited supply of drugs available be divided between expanded access programmes and clinical trials aimed at gathering the data necessary for accelerated approval?

Although there was no formal vote, the consensus from both panel and advocates in the audience was that the trials needed for approval should take precedence, thus leaving smaller quantities of the drug for expanded access, and creating the need for a group to make tough decisions about who should receive what little is available.

Roche, which is the furthest ahead in its development work and has already agreed to supply 4,000 people with its protease

inhibitor through expanded access, welcomed the decision. "We don't like playing God," said a spokesman for Roche, "so if decisions can be made jointly, we'd be happier." Merck, has so far agreed to provide drugs to 150 people, and Abbott, the newcomer, is still discussing with AIDS advocates what it will supply.

One proposal supported by AIDS activists both on the task force and in the audience was that the Food and Drug Administration (FDA) should have regulations requiring companies applying for accelerated approval to have an expanded access programme in place. And those regulations should provide guidelines for the nature of the expanded access programme, "We don't want to spend months negotiating every time there is a new drug," said Jules Levin, an advocate from San Francisco who represents people with CD4 counts lower than 200.

Expanded access, however, poses problems for industry. The company cannot make a profit on drugs supplied through expanded access (they are not approved). They can recover their costs, but with difficulty. And there are other problems. Although the FDA must agree to a drug being available through expanded access programmes, there is a legal risk in supplying a drug that is not fully approved and which is backed by incomplete safety data.

Stephen Carter, senior vice president for worldwide clinical research and development at Bristol-Myers Squibb (manufacturers of a different class of antiviral, ddI), said after the meeting: "We spent a lot of money when ddI was released on expanded access, and we were very nervous. We were moving on data from only 92 patients."

Helen Gavaghan

UK signs agreement with South Africa on joint projects

Cape Town. An 'enabling' agreement to collaborate on joint scientific projects was signed in Cape Town this week by David Hunt, Britain's science minister, and his South African counterpart, Ben Ngubane, Minister for Arts, Culture, Science and Technology.

Under the agreement, a joint committee will be set up to advise the two governments on how collaboration can best be achieved. Both South Africa and Britain will contribute £100,000 each for three years to a new fund established to support joint scientific research projects.

Further contributions to the fund are being sought from the private sector. In addition, a medal worth £10,000 will be awarded for the best collaborative project between researchers in the two countries.

During a visit designed to follow up an initiative launched by John Major, the British Prime Minister, during a visit last September. Hunt attended colloquia on agricultural research at the University of Stellenbosch, and on science and the environment, at the Council for Scientific and Industrial Research in Pretoria. He handed over equipment to the South African Astronomical Observatory at Sutherland in line with an agreement with the UK Particle Physics and Astronomy Research Council.

Conspicuously absent from the proceedings was Mrs Winnie Mandela, South Africa's Deputy Minister of Arts, Culture, Science and Technology, who is attending a film festival in Burkina Faso. Mrs Mandela defied orders from Nelson Mandela, President of South Africa and her estranged husband, to remain in South Africa and meet her commitments. It is widely expected that he intends to dismiss her when she returns on 6 March.

Michael Cherry

...as companies plan combination trials

Washington. The so-called 'Inter Company Collaboration' of US pharmaceutical companies this week begins the first of a series of small clinical trials designed to evaluate the relative effectiveness of different combinations of antiviral drugs against AIDS.

Initially, the trials will include only combinations of the AZT types of antivirals, but as protease inhibitors work their way down the pipeline, these too will be added to the combination trials.

But the design of these and other types of clinical studies drew criticism last week from AIDS activists, concerned that the kind of information that they are seeking cannot be extracted from the

trials as currently designed.

The criticisms, expressed at a meeting of the National Task Force on AIDS Drug Development (see above) prompted an irritated response from Edward Scolnick, president of the Merck Research Laboratories and a member of the task force. "Merck does know how to design clinical trials," he retorted at one point.

But David Kessler, commissioner of the Food and Drug Administration, agreed to back a workshop looking at the design of clinical trials. This will be organized by Peter Stayley, a task force member from the Treatment Action Group in New York and David Figel, director of FDA's division of antiviral products. **H.G.**

Superconductivity deal opens path to peace

Boston. The first commercial agreement stemming from an ambitious effort by the United States to curb the spread of nuclear weapons and related technology in countries of the newly-independent states (NIS) of the former Soviet Union was signed last week by Sandia National Laboratories in Albuquerque, New Mexico, ELTECH, a research institute in St Petersburg, Russia, and Superconducting Core Technologies (SCT) of Golden, Colorado.

The idea is the brainchild of Senator Pete Domenici (Republican, New Mexico), who sponsored legislation creating the Industrial Partnering Program (IPP). This programme, which is administered by the Department of Energy (DoE) and started last June, supports research collaboration between DoE laboratories and their NIS counterparts.

The aim is to engage NIS weapons scientists and engineers in developing civilian, commercially-orientated technology, in order to reduce their inclination either to continue to work on nuclear weapons domestically or to take their skills to another aspiring nuclear power. It is also hoped that the joint ventures will result in marketable products with the help of the US Industrial Coalition (USIC), an alliance of 76 compa-

nies formed under IPP.

"Until now, no one had thought of involving US industry directly in the non-proliferation process," says John Hnatio, the programme manager for IPP at DoE. "I'm convinced that this is the best national security investment this country could make." Michael Deegan, president of USIC, says the venture is "peace on the cheap".

More than 200 research projects have so far been initiated in fields as diverse as materials processing, renewable energy technologies, environmental cleanup and waste management. US laboratories nurture these small-scale research and development projects through an incubation stage, explains Dennis Croessmann, programme manager at Sandia Laboratories. Officials then select those that appear to be the most commercially viable and try to interest outside companies.

The two-year contract with ELTECH and SCT involves developing tunable microwave filters made with high-temperature, superconducting thin films and ferroelectric materials to improve communications on cellular phones. "SCT had already been talking with us and the Russians before IPP was even in place," explains Duane Dimos, who heads

the Sandia effort. The relationship, he says, combines ELTECH's strength in microwave components with Sandia's expertise in ferroelectrics and SCT's marketing knowledge.

"This first agreement was probably the toughest, given the differences between the US and NIS systems," says Croessmann. "Now that we've been through it, the next ones should be a lot easier." Twenty more commercialization agreements are close to being signed, with 10 more pending.

Participants are enthusiastic about the flood of new ideas. "There's been a wall between these countries for decades," Croessmann says. "Now we have the chance to tap into a motherlode of technology across a broad range of research areas."

William Collins, vice president of USIC, accepts that there is a 'bonanza' waiting to be tapped — but warns that it may be difficult to reach. "We help companies learn about new opportunities and we share in the investment cost"

In courses taught at 13 universities, US scientists and business people learn about various aspects of doing business with the NIS. At the same time, NIS scientists learn how to commercialize and market their technology.

Steve Nadis

European nations to collaborate on flood research

Munich. Four European countries have agreed to work together, in collaboration with the European Commission, on developing research programmes in response to the recent Rhine floods.

The proposed programmes will focus on modelling the basins of the two rivers whose flooding poses the biggest threat to northern Europe, the Rhine and the Meuse. The international collaboration follows an initiative by the Dutch minister of science, who is particularly concerned at the way in which the flooded river threatened to burst his country's dikes.

The past century has seen a significant change in response of the Rhine to heavy rains. Hydrological records show that, 100 years ago, increases in river flow after heavy rains were spread over eight to ten days. Today the extra flow is concentrated in little more than half that time.

One reason has been the straightening of the river to make it more navigable, and its separation by dikes from the flood plains that previously buffered peak flows. Climate change may also be a factor because of its impact on rainfall.

A few days after the floods at the end of January, the environment ministers of France, Germany, Belgium, Luxembourg and the Netherlands issued a joint statement describing as "unacceptable" the risks to



Up to the brim: Flood barriers in Cologne

"life, property and the environment" of frequent severe flooding. They called for broad international measures on the implications of land use and water management.

In a separate move, the Dutch research minister, Jo Ritzen, hosted a meeting in Maastricht on 16 February, with representatives from Germany, Belgium and the commission, to discuss international collaboration in research on flooding.

The commission agreed to support a workshop in April in Brussels to develop research proposals for modelling the Rhine and Meuse, and for improving understanding of dyke construction and dike failure.

The commission is already sponsoring a series of expert meetings to prepare for the workshop, and wants to bring more com-

plete models of the rivers into operation within two years, to give the Low Countries a longer period of flood warning. The eventual objective of the research, says a commission spokesman, is to provide government authorities in the countries that cooperate through the International Rhine Commission with information to help to deal with future floods.

Two Dutch institutes brought proposals to the meeting that will serve as a basis for developing collaborative programmes with institutes in the other states. The Delft Hydraulics Laboratory wants to collect data from the spans of river that have already been modelled, and fill in the gaps. The Delft Geotechnics Laboratory is planning to examine the response of dikes to severe loads; it is particularly interested in the situation caused by two floods in close succession, where the second must be contained by dikes already fully saturated.

"There is no simulation model of the whole of the Rhine basin and this is what we need if we are to get more advanced predictions, maybe one or two days ahead", says Peter Tindemans of the Dutch ministry of education and science. "Furthermore, we don't have enough information about the conditions of all the dikes because many are so old."

Alison Abbott

Wellcome Trust to launch transfer company

London. The Wellcome Trust is planning to set up a technology-transfer company to help the scientists it funds to find commercial outlets for the results of their research.

According to Bridget Ogilvie, director of what is expected to become the largest private medical research foundation in the world (see below), the decision has been prompted by anticipated guidelines from the Charity Commissioners emphasizing that charities have a duty to ensure that the research they finance is properly exploited.

But Ogilvie says that the move has also been prompted by complaints from many Wellcome-funded scientists in universities about the lack of adequate support from the technology-transfer mechanisms set up by the universities for which they work.

Although the details of how the new company will operate are still being worked out,

Julian Jack, the chairman of the trust's scientific committee, told a parliamentary committee last week that its goal is "to stop the work it supports from being buried."

Jack was answering criticism by Sir Gerard Vaughan (Conservative, Reading) that the trust was seen in some quarters as concentrating excessively on fundamental research, and neglecting the follow-up needed to make the results of the research it funds available to society.

"We have been rather slow off the block," admitted Jack. "We now accept that we have a responsibility to help scientists get their discoveries patented and exploited, and intend to do this by setting up our own technology transfer company; but we are not doing this to make money."

Various possible mechanisms have already been discussed within the trust. At

one point, for example, detailed consideration was given to an agreement with CRC Technology (CRCT) — the licensing arm of the Cancer Research Campaign — under which the company would carry out licensing arrangements on behalf of the trust.

More recently, an alternative plan has been explored that would involve a joint venture between the trust and CRCT. According to Jack, the role of any such body would be essentially "to act in an advisory capacity". Moving in this direction would require the trust to make some constitutional changes. "But we hope and expect that we will have no difficulty with that."

Wellcome's move has been widely welcomed in both the academic and industrial research communities who have been wanting it to take a more active role in promoting the applications of its research. This pressure has been increasing over recent years with the realization that much of the fundamental research that Wellcome funds in human genetics, for example, has potentially wide-ranging (and profitable) applications in developing new diagnostic and therapeutic techniques.

Ogilvie says that, until recently, the trust's position on patenting and intellectual property was that this was a matter for the universities.

"We changed this because the scientists we funded in universities were begging us to help them," she says. "They found that the local support was not there."

Not all universities, however, are happy about Wellcome's plans. Some claim that,



Ogilvie: Reacting to scientists' requests

by making more explicit demands about the way in which its scientists should handle intellectual property, the trust is treading on the universities' toes, ignoring the experience they have built up in technology licensing over the past ten to 15 years.

"We are concerned that Wellcome's attempts to take greater control over patent rights is likely to restrict the freedom of universities to use research results in the way that they see fit," says Adrian Hill, director of industrial liaison for the University of Bristol, and chairman of the University Director of Industrial Liaison.

But Ogilvie says that the amount of money required to operate successfully technology transfer is such that many universities (like most charities) are not in a position to do so. Wellcome is in the fortunate position of being able to provide the necessary level of funding, she says. **David Dickson**

Glaxo move seen as 'unavoidable'

London. The Wellcome Trust — the UK charitable body set up to hold the shares of the Burroughs Wellcome pharmaceutical company on the death of its founder, Sir Henry Wellcome in 1936 — was told by its lawyers that it would be "virtually illegal" not to accept an offer for its outstanding 40 per cent holding in the company to Glaxo, according to the chairman of the trust's scientific committee, Julian Jack.

The decision was taken even though the sale of the company now known as the Wellcome Foundation will inevitably lead to substantial job losses among its research and other staff, whose interests the trust is required to protect in Wellcome's will, as well as a reduction in spending on pharmaceutical research in Britain.

"We had to take advice, and it was clear what our fiduciary duty was," Jack said last week in the trust's first public statement on the proposed take-over since Glaxo's bid was announced on 23 January (see *Nature*, 373, 271; 1995). "Whatever our regrets, we were persuaded that we would be behaving virtually illegally if we did not accept."

Jack was presenting evidence to the House of Commons Select Committee on Science and Technology, whose members expressed concern at the net reduction in research and development spending likely to result from a rationalization of the two companies that has been promised by Glaxo's chief executive (and former research director) Richard Sykes.

"Our worry is over the impact that this move could have on the overall science base of the country," said the chairman of the committee, Sir Giles Shaw.

The chief executive of Wellcome, John Robb, who said that he had been "surprised" by the Glaxo bid when it arrived on

the morning of Monday, 23 January, told the committee that he shared its concern.

He pointed out, for example, that the increased funding for biomedical research through the Wellcome Trust — estimated at between £50 million a year — was likely to be more than offset by cuts Glaxo is planning to eliminate duplication in research and development by the two companies.

"The consequence [of] narrowing of the UK science base will not only have a devastating effect on those scientists who lose their livelihood, but is likely to have an additional negative impact on those currently considering a career in the biomedical sciences," said Robb.

Sykes, in contrast, defended the planned merger, and subsequent rationalizations, as essential if the new company, which will become the largest pharmaceutical company in the world, is to remain successful. Sykes did not deny that the savings he is planning to make by merging the research staff of the two companies are likely to lead to significant redundancies. (Wellcome calculates that up to 4,000 research and development staff may lose their jobs.) "But the reason for doing it is to create a situation in which there may be more jobs at the end of the day and not less," he said.

Speaking on behalf of the Wellcome Trust, which as a result of the new injection of capital will become the largest private medical research foundation in the world, Jack said that the trustees had discussed the likely impact of Glaxo's bid on Wellcome's employees. "We were told that it would be outside our fiduciary duties to set any conditions, and we had to accept that advice," said Jack. "But when Richard Sykes came and talked to us [about Glaxo's plans], we did discuss our concerns with him." **D. D.**

Waste shipment stirs debate over lack of nuclear store

Tokyo. Japan's shipment of high-level radioactive nuclear waste from France, which left in the face of environmentalists' protests last week, has drawn attention to the lack of a permanent site to store such waste material.

The British ship *Pacific Pintail* is returning to Japan the first 28 of 3,000 cylindrical containers of high-level waste derived from reprocessed Japanese nuclear fuel that must be returned to Japan from reprocessing plants in France and Britain over the course of the next decade.

At the same time, the 47 nuclear power plants operating in Japan are generating the equivalent of about 1,000 further containers of such waste every year. But the government and nuclear power industry has no permanent disposal site for the waste.

That being carried by the *Pacific Pintail* takes the form of glass contained in cylindrical stainless steel containers, each 1.3 metres in length and 43 centimetres diameter. The containers will be "temporarily" stored for between 30 and 50 years in a high-level waste storage facility at Rokkasho village on the northern tip of Japan's main island, Honshu.

Rokkasho is the site of a multi-billion dollar complex for uranium enrichment, for the storage of both low-level and high-level waste, and for nuclear fuel reprocessing. All the facilities are complete except the fuel-reprocessing plant, which is expected to begin operation early next decade, after several years delay (see *Nature*, 369, 596; 1994).

The nuclear industry and the government have jointly won local support for the Rokkasho complex partly by providing massive government subsidies to the village amounting to ¥18,000 million (\$180 million), or about ¥150,000 for each of its 12,000 inhabitants (see *Nature*, 345, 285; 1990). But the villagers remain opposed to the permanent storage of high-level waste at Rokkasho.

Long-term government plans call for the waste to be buried at a depth of more than 500 metres. But government and industry have yet to find a suitable site.

The waste will remain highly radioactive and toxic for thousands of years and the disposal site must be stable for this length of time. But on this timescale, nearly all regions of Japan are threatened by earthquakes and/or volcanic eruptions.

A more immediate problem is that no local community in Japan will accept a permanent waste disposal site. Even attempts to carry out research on disposal are bitterly opposed by local residents.

In 1985, for example, when officials of the government-owned Power Reactor and Nuclear Fuel Development Corporation

(PNC) tried to carry out a survey at Horonobe, a small town in the northern island of Hokkaido, they were blocked by hundreds of protesting residents and union members, who feared that Horonobe would become a permanent disposal site.

PNC officials had to sneak onto the site at the weekend, when the local residents had relaxed their guard, in order to choose sites for boreholes and collect rock samples.

PNC maintains that Horonobe is not a candidate for permanent disposal of waste and that, at present, their only purpose at Horonobe is to carry out research on disposal. "There are no candidate sites at present," insists a PNC official.

A decision on a site is still a long way off. The long-term plans of the Atomic Energy Commission released last year only call for establishment of an organization to handle high-level waste disposal by 2000; actual disposal would begin between 2030 and the middle of the 2040s.

Meanwhile, the government is pursuing another route to try to reduce the amount of high-level waste that must be disposed of. In the budget for fiscal year 1995 (starting on 1 April), the Science and Technology Agency has ¥1,364 million (\$13.6 million) for PNC to embark on a long-term programme to develop a new form of nuclear fuel reprocessing (see *Nature* 359, 766; 1992).

This procedure would incorporate into reprocessed plutonium fuel highly radioactive minor actinide elements such as americium, neptunium and curium that are normally discarded as high-level waste.

PNC estimates that if this technique was applied and the fuel repeatedly burned and recycled in all fast breeder reactors that are expected to begin commercial operations from 2030, the amount of minor actinides produced by Japan's nuclear power industry by 2100 would be reduced from the 310 tons expected from present reprocessing techniques to 60 tons. Furthermore, nearly all of the 60 tons would be locked into repeated fuel cycles of the fast breeder reactors rather than being in the form of waste.

The government sees another advantage in this approach. The plutonium fuel containing minor actinides will be unsuitable for diversion to the manufacture of nuclear weapons. It might therefore help to reduce international concern that Japan might use its growing stockpile of plutonium to make nuclear weapons.

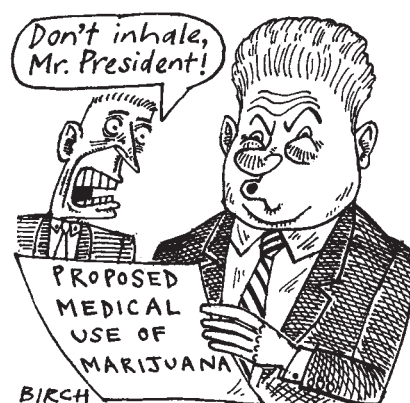
The catch is that such fuel is much more radioactive than pure plutonium, and will need to be made and handled by specially developed robots. PNC officials do not expect their project to be ready for practical application for at least 10 to 15 years.

David Swinbanks

US stalls over tests of marijuana to treat AIDS patients

San Francisco. US drug enforcement policy has collided with scientific freedom over a study of the potential medical use of marijuana, delaying research into what some claim could be a life-saving therapy for AIDS-related wasting.

Donald Abrams of the University of California, San Francisco (UCSF), has been trying for more than two years to obtain approval for research into whether marijuana can restore the appetite and body weight of individuals with AIDS-related wasting. His study is to be sponsored by the Community Consortium on AIDS Research, of which he is research director, and would be the first officially



approved trial in the United States evaluating the potential medical benefit of marijuana.

Yet even though the protocol for the trial has been accepted by the California Research Advisory Panel and the UCSF Review Board, as well as by the Food and Drug Administration (FDA), Abrams has been unable to obtain a supply of the drug because of a ban by the Drug Enforcement Administration (DEA) on importing marijuana, and the refusal of the National Institute of Drug Abuse to provide it. A meeting called by the US Public Health Service to discuss the problem has been postponed twice and never rescheduled.

Supporters of marijuana use think Abrams has run up against deliberate government opposition. His critics feel that he has become a pawn of those wanting to legalize the drug. But other researchers into the physiological effects of marijuana wonder why he is being treated differently from them.

One government official confirms what many observers suspect: that agencies determined to convince the public that marijuana is dangerous are not keen on a study that could show some beneficial effect. Indeed, the Clinton administration is said to be reluctant to champion a study

EPO verdict lifts patent coverage of seeds

Munich. The highest appeal board of the European Patent Office (EPO) has ruled that a patent granted to the Belgian company Plant Genetic Systems and the US biotechnology company Biogen Inc. on a procedure for producing herbicide-resistant plants through genetic engineering cannot cover the plants and seeds resulting from this process.

Although plant varieties are excluded from patentability under the terms of the European Patent Convention of 1973, this is the first time that the EPO has explicitly restricted a patent from covering a specified plant. This could have a major impact on the scope of the 100 or so patents already granted by the EPO on other genetically engineered plants — although the full impact will not be known until the EPO publishes the reasons for its decision in a few weeks.

Greenpeace, which had opposed the patent, is already claiming that the decision represents a significant victory for those who believe that life forms should not be patentable. But the plant biotechnology industry says that it is not unduly worried by the ruling; Plant Genetics Systems, for example, says that the decision will not in practice reduce its patent protection.

In 1991 Greenpeace challenged its patent, which covers a process whereby plants can be made resistant to the herbicide Basta, a glutathione synthetase inhibitor

manufactured by Hoechst. The patent covered the sequence of the gene which expresses an enzyme that deactivates the herbicide, and its vector, as well as the plant cells in which the gene is expressed, and both plants expressing the gene and seeds containing it.

Greenpeace argued that the patent contravened the article in the European Patent Convention stating that patents may not be granted for "plant or animal varieties". This argument was initially rejected by the EPO's opposition division. But in a ruling last week its technical board of appeals accepted some of Greenpeace's objections, and the patent holders agreed to drop their claims for protection of plants and seeds.

At issue is the precise interpretation of the article in the convention forbidding patents on plant varieties. According to earlier EPO rulings, plants themselves are patentable because they are not excluded by this clause. But the EPO now appears to have changed its interpretation.

According to Jan van Rompaey, patent manager of Plant Genetic Systems, the appeal board may have concluded that, by definition, the term plant encompasses a group of plant varieties, and is therefore unpatentable.

According to the 1991 definition drawn up by the International Union for the Protection of New Plant Varieties (UPOV),

linked to the World Intellectual Property Organization, a United Nations body in Geneva, a plant variety is the smallest unit displaying uniform characteristics that can be distinguished within a species. "As an example," says André Heitz, a spokesman for UPOV, "'apple' denotes a plant species, but 'Golden Delicious' is a variety."

Plant varieties were excluded from patents under the convention because the creation of a plant variety by simple breeding was not then considered by many countries to involve a true inventive step. Exclusive protection of plant varieties was provided by the UPOV convention.

But the advent of gene technology, which introduced the possibility of creating new plants and thereby new plant varieties, by a truly inventive step, rendered the exclusion paradoxical.

Plant varieties are explicitly excluded from patenting under a proposed European Union directive on the protection of biotechnological inventions, which is being voted on by the European Parliament this week (see *Nature* 373, 550; 1995). The proposed directive states that "biological material, including plants and animals, as well as parts of plants and animals, except plant and animal varieties as such, shall be patentable".

It further states that "the protection conferred by a patent on a product containing or consisting of genetic information shall extend to all material in which the product is incorporated and in which the genetic information is contained and expressed".

Dominique Vandergheynst, who helped draw up the directive, says that if the directive is accepted, and consequently brought into national legislation, it will be hard for the EPO to argue that plants cannot be patented. In the future, he claims, the EPO will need to abandon its clause disallowing the patenting of plant varieties, as the basis for the distinction is no longer valid.

But he emphasizes that until the EPO's written ruling is available for study, it should not be assumed that the office has in fact assumed a position contrary to the directive. The ruling could be simply a consequence of loose wording in this particular patent, he says, whereby perhaps the description of 'plants' could be interpreted as a description of 'plant variety'.

Surprisingly, industry claims not to be concerned about last week's verdict. Tim Roberts, chairman of the biotechnology committee of the Chartered Institute of Patent Agents, and official spokesman for the senior advisory group on biotechnology to the European Chemical Industry Council, says that because claims for plant cells expressing the herbicide-resistant gene have been allowed, the overall patent protection is not diminished.

Alison Abbott

(from previous page) that, despite its scientific merits, could open the president to attack for supporting an illegal drug.

Wasting is a leading cause of death for AIDS patients and affects about 25 per cent of those whose immune system capacity has dropped into the 200 T-cell range. But many people who have tried Marinolan pills, an FDA-approved drug containing THC (tetrahydrocannabinol) the primary active ingredient in marijuana, say it either does not make them want to eat or it makes them so intoxicated they cannot think. Instead they turn to smoking marijuana.

Abrams started his campaign in 1992 when 'Brownie Mary', a 73-year-old San Francisco General Hospital volunteer, was arrested in nearby Sonoma County (north of San Francisco) for supplying AIDS patients with marijuana-laced brownies. With support from activists promoting the medical use of psychedelic drugs, Abrams decided to conduct a proper study.

He plans to compare the appetite-stimulating effects of three doses of marijuana smoked with a water pipe to Marinol pills. The study would involve 40 people, measuring their hunger and weight gain, as well as the effects on their lungs, immune systems and the level of HIV in

their bodies.

But US state and federal governments disapprove of the smoking of marijuana for medical purposes. Pete Wilson, the governor of California, vetoed a bill last September allowing medical use of the drug. A month later, the federal government decided to maintain its ban.

Those who prefer to smoke the drug rather than take THC say that smoking is cheaper, that it is easier to control the effect on their mental capacity, and that the complex herbal compounds stimulate their appetite more than the pills.

But Abrams is still waiting for a licence from the DEA to dispense marijuana, which is rated a Schedule I substance along with heroin and LSD. He must also obtain supplies of the drug, which will require either permission from the National Institute on Drug Abuse (NIDA), or an import licence for HortaPharm, a Dutch company that Abrams says has agreed to supply him with a \$50,000-research grant, as well as all the free marijuana he needs.

Government officials accept that the use of marijuana to boost appetite merits study in part because it is relevant to an important cause of death among AIDS patients.

Sally Lehrman

Protecting wildlife becomes endangered act

Washington. More than 20 years after becoming law, the US Endangered Species Act (ESA) faces major challenges in the Congress and Supreme Court that could severely limit its ability to protect threatened species. With the act scheduled for reauthorization this year, conservative Republicans in Congress have vowed to amend it to increase protection not of wildlife, but of private property owners.

Don Young (Republican, Alaska), chairman of the House of Representatives Resources Committee, plans to introduce an ESA reauthorization bill by early summer that will almost certainly call for landowners to be compensated by the government if their property loses value as the result of an endangered species listing. Opponents of the current law say that such losses amount to an illegal 'taking' of private property by the government.

Protection of property rights, and hostility toward federal regulations, are major themes for the new Republican majority, which last week pushed a measure through the House freezing most new federal rule-making for the rest of this year — or until legislation overhauling the entire regulatory process is passed.

That process begins this week, when the House votes on a sweeping package that calls for 'takings' compensation, along with additional layers of review and risk assessment before any new federal rule is issued. Even if the Senate dilutes these bills, the takings issue is likely to surface with every environmental law that comes before Congress in the next two years.

Young has appointed a task force chaired by Richard Pombo, an arch-conservative Republican who is also a California rancher, to look into the reauthorization. The task force will hold public hearings beginning next month for citizens around the country. Similar hearings are planned by Dirk Kempthorne of Idaho, who chairs the equivalent Senate subcommittee.

All three congressmen have been vocal critics of the act, and the meetings are expected to provide a forum for landowners — primarily in Western states — to vent their anger at what they see as excessive federal regulation.

Meanwhile, bills have been introduced in both the House and Senate that would halt any new species listings or designation of

those burdens on small landowners.

With this in mind, the Interior Department recently introduced new policies designed to make it easier for property owners to comply with the act. For example, landowners with an endangered species on their property who agree to a long-term conservation plan will no longer be asked to commit more money or land at a later date if the needs of the species change.

The US Fish and Wildlife Service (FWS), which administers the act within the Department of Interior, has also requested additional money in 1996 to help states to develop habitat conservation plans. But these steps may not be enough to appease Republican critics, who have held up the ESA as one of their prime examples of intrusive government.

In attacking the current law, Republicans are also calling for more stringent scientific review of listing decisions for endangered species. "We need to bring sound science into the equation," says Kempthorne. "The listing of a species should be based solely on science."

But the FWS is already required to solicit extensive comment, including a recently instituted peer review by three outside scientists, for any listing decision. And even though many scientists say there are flaws in the current listing process — for example, its overemphasis on high-profile vertebrates — the basic rationale is generally considered sound.

Environmentalists therefore charge that the demand for more review is simply a ploy to introduce delays or to allow non-scientists — including landowners — to influence the listing process. And they question why some Republicans want to strangle the fledgling National Biological Service (NBS), when that agency supplies the very scientific advice Kempthorne has requested. A House subcommittee last week voted to rescind \$17 million from the NBS, keeping the agency barely at maintenance level.

Budget cuts may in fact be the most effective way for Republicans to weaken the ESA even before it is reauthorized. The same House panel rescinded \$2 million from this year's FWS budget for listing new endangered species. That allows about 100 new listings this year — the minimum required to comply with a legal settlement stemming from a lawsuit by environmental groups. Some 170 listings had been planned in 1995.

As well as these attacks in Congress, the ESA faces one more hurdle this spring. The US Supreme Court has agreed to hear in April the case of Sweet Home Chapter of Communities for a Great Oregon v. Babbitt, which will determine whether the destruction of a species' habitat can be considered 'harm' under the ESA. **Tony Reichhardt**

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Under threat: the black bear is one species whose future is hanging in the balance

new critical habitat until the ESA is reauthorized. More than two dozen Republican Senators sent a letter in late January to chairman of the Senate environment committee, John Chafee of Rhode Island, pressing for early hearings on the bills.

In such a charged atmosphere, environmentalists and Democratic protectors of the ESA have been girding themselves for battle. But some moderates, such as Chafee, are searching for a middle ground. He and Bruce Babbitt, the Interior Secretary, have argued in recent weeks that property rights and protection of species are not mutually exclusive, and that claims of lost property value due to endangered species listings have been exaggerated. They concede, however, that the ESA does impose financial burdens in certain cases, and that the federal government could do a better job of easing

French culinary secrets examined

Paris. Not content with having a University of Oenology, in Bordeaux, for the study and application of 'wine science', France is now to create a European Centre for Taste Sciences at Dijon, home to the famous brand of mustard.

The idea of the centre has been cooked up by the food group Danone, the University of Burgundy, and the Centre National de la Recherche Scientifique (CNRS). Danone says it wants to create a "world-class" centre of research in a field with "considerable economic and social consequences".

Construction of the centre, which is expected to employ 80 staff, will begin this year. It will include an institute for fundamental research on taste, and a foundation designed to raise both awareness of and funds for taste research.

The creation of the centre's research recipes will be overseen by Styliano Nicolaidis, a CNRS researcher who works at the Collège de France in Paris. On the menu will be physiology, the modelling of olfaction, the neurophysiology of 'degustation' ('tasting'), and the social science of taste. *Bon appetit!* Declan Butler

Truth is the daughter of time

SIR — John Godfrey (*Nature* 373, 100; 1995) attempts to reason with the Pope about human ontogeny. Reason does work with the Roman Catholic Church, but the timescale is the problem. It took 400 years for the Church to admit the truth about Galileo. How long will it take it to admit it is wrong about human reproduction?

My wife and I recently visited Mother Teresa's orphanage in the ghastly slums of Calcutta, and met children who were a small and lucky fraction of the unwanted flotsam of that city of dreadful night. That evening we saw a BBC report about the Pope's fulminations in Manila on the evils of birth control and family planning. The contrast was wrenching.

What is happening in Calcutta would seem at first sight to be irrelevant to the Church's attitude. The parents of these children are (supposing that they have any time for religion) Hindus or Muslims or Jains or whatever. Yet imagine that 30 years ago Pope John XXIII had lived long enough to change the Church's teaching on birth control. Who can doubt that this decision would have resounded through the rest of the religions of the planet, and through the halls of governments everywhere?

The fear is that when, decades or centuries from now, some future Pope changes his mind, this belated decision will be as irrelevant as the Church's "vindication" of Galileo. The difference is that this decision matters. Every year that it is delayed makes it clearer that the Church's current attitude is a crime against humanity. History will judge it so.

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SIR — It is unfortunate that Godfrey missed seeing the forest because of attention paid to the trees. It is also unfortunate that he should berate the Pope for discussing a "moment" when an individual human life begins as being contrary to modern biological knowledge. The establishment of totipotency following fertilization is a well-established fact that is fully consistent with the Pope's criticized discourse. Why quibble over whether the time elapsed is appropriately referred to as an instant or a moment?

The Pope proclaims that every human is precious, a pearl of great price. Yet modern biological knowledge cannot justify ascribing dignity to any human. Science is incapable of preventing a total loss of any sense of human dignity in contemporary culture. A society that rejects equal dignity for all humans is the definition of tyranny. This is a concern shared by

people of many faiths, including the Pope. Those who look to science to provide a reason for human dignity will be eternally disappointed. Let us not seek out those humans whom we can feel justified in denying their rights, rather, let us cherish each person.

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SIR — I wonder what my students would think if, at the start of my lectures on the cellular aspects of developmental biology, I brainwashed them with Godfrey's slogan that "there is no moment when human life starts". Developmental biology emphasizes the starting point of every new organism. We know well the cellular traits, molecular composition, regions, potentialities and even fate maps of the first cell, the zygote, of flies, sea urchins, nematodes and frogs. Why should human biology be completely different?

Godfrey does not offer any evidence to discredit the long-standing and 'common sense biology' view supporting fertilization as the standard origin for the human individual. It is not "obsolete biology" but a mere description of facts. Even recognizing the complexities of fertilization, its duration included, simple observation shows us that, as with any other animal species, fertilization is the moment when an individual human organism starts its development. The widely used concept of developmental age relies in being able to identify a beginning. If the conclusion of the fertilization process marks the start of every new specimen of *Drosophila*, *Caenorhabditis*, *Xenopus*, *Gallus* or *Mus*, the end of fertilization should also be considered the origin of a new human being.

It is not John Paul II who is trying to impose a non-scientifically based description of human first developmental stages. If anybody is trying to force the scientific facts, it is Godfrey. Personally, I cannot offer my students the farce of human embryos without beginning. I prefer telling them that we are having a hard time dealing with the early human embryo: that besides those who respect him/her as a human being, there are others who, in order to justify the deliberate loss or destruction of human embryos, prefer to hold them as not yet clearly defined entities. The pretence of indeterminate origin is simply an element of this scheme.

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Race plus IQ does not equal science

SIR — Steve Blinkhorn's recent review (*Nature* 372, 417–419; 1994) of Herrnstein and Murray's *The Bell Curve* (and two related books) misses the point. Considered as scientific inquiry, the basic question posed by the authors — is a social "underclass", which performs poorly on IQ tests, doomed to this deficiency by the genes of its members rather than by remediable epigenetic factors? — is unanswerable. The reason for this conclusion is that the list of epigenetic influences that might give rise to the differential behaviour of any large and heterogeneous group of human beings is, for all practical purposes, limitless.

A problem in biology that underlines this point is cell determination. Developing cells are said to be 'determined' when varying the circumstances in which they exist fails to influence what they become. Much pointless argument has been expended over the years because of the erroneous assumption that if a cell's properties are demonstrably stable under some set of testable conditions, its fate is sealed for all conditions. But there are of course always other circumstances that, had they been tested, might have altered a cell's fate. Although the question of cell determination is in principle amenable to analysis, most biologists have avoided it, recognizing the wisdom of Peter Medawar's dictum that success in science depends on studying problems that can actually be solved (*The Art of the Soluble*, Methuen, 1967).

The role of genes in the determination of IQ (save in the case of monozygotic twins) is doomed to a similar scientific limbo, and should likewise be dropped as a serious issue. Whereas intelligence is in part heritable (as all and sundry admit), it is simply impossible to determine that any group owes a small difference in performance to 'genetic factors'. There will always be other untested (or untestable) epigenetic influences that might explain the discrepancy, as must be especially obvious in the case of 'race' (whatever that means) and IQ. Were the thesis of *The Bell Curve* not so pernicious, it would be sufficient to let it die a natural death. However, the unfortunate consequences of having so many people, including scientists, take seriously the highly implausible idea of genetically determined racial inferiority is by now obvious. A problem that cannot be solved is not the stuff of science, but of polemics.

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Towards unequal partition of energy?

A tantalizing simulation of simple colliding particles in one dimension reveals a clumping tendency in which energy is by no means equally shared among them. The bearing of this on three-dimensional hydrodynamics is another matter.

Now for something conceptually simple, the problem of relating the macroscopic behaviour of some object to the behaviour of its constituent parts, ultimately of the atoms of which it consists. Versions of the problem are well rehearsed in the most elementary of physics textbooks.

The shape taken by a string suspended between two points and hanging freely under gravity? Simple; a piece of string, by definition, can exert a force on the piece to which it is attached only along the line joining them, which is the tangent to the familiar catenary curve. And the same with the usual conundrums of bending beams; on the assumption that the applied forces are not so great that the longitudinal integrity of the structure is destroyed (or that the beam does not snap), the forces to which one part of the beam is subjected consist of torques or bending moments exerted by its neighbour pieces and by the downward force of gravity.

Classical physics, in other words, is a way of representing the microscopic forces between atoms and molecules with plausible patterns of macroscopic forces. That, for example, is the case for the simple calculation of the vibration of a string under tension, or of the velocity of sound in a gas (where it is simply necessary to know the equation of state and the ratio of specific heats at constant volume and pressure respectively). Then, simple calculation will yield a plausible result. Hardly anybody would dream of tackling such a problem by starting with a distribution function for the position and velocity of the constituent molecules and then allowing for their mutual collisions (but, among other excellent things, Chapman and Cowling did just that in their classical monograph, *The Mathematical Theory of Non-Uniform Gases*).

Hydrodynamics more generally is based on the same principles. People speak of infinitesimal elements of fluid, which are supposed to obey the laws of mechanics. Because they have internal structure, the same particles are allowed to have thermodynamic properties as well. (The velocity of sound in a gas is a particular case.) Frictional forces are allowed, and lead to the dissipation of energy. But how generally valid is the supposition that microscopic behaviour can be described in macroscopic terms?

Leo P. Kadanoff from the University of Chicago, with Yunson Du and Hao Li, has now, a little subversively, set out to demonstrate that there are exceptions to

the rule by means of one of the simplest systems there could be — a set of classical particles moving in one dimension in a simple confining box, capable of colliding with each other and in such a way that energy may be lost in the process (*Phys. Rev. Lett.* **74**, 1268–1271; 1995). The objective is to follow the collisions of the particles individually and to compare the behaviour of the system they constitute with the appropriate macroscopic hydrodynamic equations.

A deliciously school-textbook notion then arises, the “coefficient of restitution”, which is a number between 0 and 1 representing the fraction of the relative velocity of two particles retained in a collision. Think of bouncing a ball vertically onto the floor; if the impact velocity is v , the velocity on rebound will be $-rv$, where r is the coefficient (and the minus sign allows for the rebound of the ball in an opposite direction).

Evidently, such a system will not remain in motion indefinitely. Because energy is lost at each collision, the point will be reached at which there is no kinetic energy left, and all the particles will be at rest. To avoid that trivial outcome, the authors specify appropriately the boundary conditions at the walls of the box. Collisions at one wall are supposed to be perfectly elastic; the rebound velocity is equal to the impact velocity, but in the opposite direction. But at the other wall, energy is fed into the system of colliding particles. This may be notionally arranged in various ways. One is to return each impacting particle into the collision space with a fixed velocity, another is to return the particle with a velocity taken from some random distribution, yet another is to suppose that the wall is vibrating and that particles collide with it elastically. Whatever the arrangement, one wall feeds energy into the system to compensate for that lost in collisions between particles.

The end result appears to be more or less inescapable, whatever the starting distribution of the particles in their one-dimensional box. Because the particles can only collide with each other, and cannot pass through one another, the particle nearest the wall that serves as a source of energy will move very quickly, but the other particles will tend to huddle near the other wall as if they were sheep penned in by dogs.

The authors argue that this condition is robust in that it is reached from all conceivable starting conditions. In the case in

which the energetic wall provides the particle reaching it with variable velocity, the particles clumped together near the other wall may burst out of their confinement, only to be returned to it when the free particle is given a greater velocity.

When both walls are sources of energy, most of the particles clump together away from the walls, but support runners on either side of them will fetch and carry energy. In the case in which the wall that is the source of energy returns all particles reaching it with a finite speed, and in which the other particles start with zero velocity, the centre of gravity of the clump moves rhythmically, and the range over which the particles within it are spread increases (and decreases) in unison.

Cynics will say that all this simulation is just a way of occupying inventive people on tasks with which they are familiar when there is nothing more useful in their minds, but that would be a mean *canard*. For the truth is that the simulations demonstrate a direct contravention of what is called the theorem of the ‘equipartition of energy’ — the doctrine that in a system of interacting particles, energy will be distributed, on the average, equally between all of them. And that is the basis of classical hydrodynamics and much else.

A further feature of the simulation is that the results are quite different when collisions between the particles are elastic (or the coefficient of restitution is equal to unity). Then, in the long run, there is no penning of the majority of particles into clumps, energy (on the average) is equally shared between all of them and the equations of classical hydrodynamics can be expected faithfully to apply.

So where is the snag? The concluding sentence of Du *et al.* is “it remains to be seen whether such a behaviour persists in a driven system in higher dimensions”. That may be the crux of this affair. The trouble with a one-dimensional system is that it provides a natural hierarchy for the interaction between one particle and its neighbours; only the next nearest matters. That, in other contexts, is the reason why long-range order cannot exist in one-dimensional systems on the basis of interactions between next-nearest neighbours alone. But it will be interesting to learn what the two-dimensional simulations now implicitly promised will show. A similar result would indeed be subversive. That outcome seems improbable, but it is important that it should be attempted. And soon.

John Maddox

Active ancestral molecules

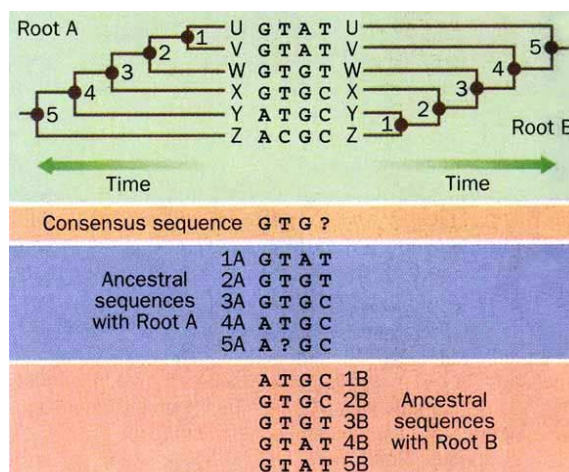
Caro-Beth Stewart

OVER 30 years ago, Linus Pauling and Emile Zuckerkandl¹ proposed that sequences of molecules from living organisms can be used to infer sequences that were present in their common ancestors. Although the technology was not then available, they envisaged that "it will in the future be possible to synthesize these presumed components of extinct organisms. Thus, one will be able to study the physico-chemical properties of these molecules and to make inferences about their functions"¹. Several reports²⁻⁴, including one by Steve Benner and colleagues on page 57 of this issue⁵, show that this future has arrived. These papers describe reconstructed 'ancestral' sequences that encode biologically active molecules, some with unexpected properties.

First, a few words need to be said about ancestral reconstruction. As illustrated in the figure, ancestral sequences are reconstructed through logical inference upon an evolutionary tree, a process referred to as parsimony analysis^{6,7}. If the modern sequences are not too divergent and the tree has enough branches, reconstructions can be made with few or no ambiguities⁸. Moreover, such parsimonious reconstructions (on correct phylogenies) seem to be largely accurate. David Hillis and colleagues⁹ have experimentally evolved bacteriophage T7 phylogenies in the laboratory and have sequenced the actual ancestors. Parsimony reconstructs the known ancestral states with 98–100 per cent accuracy (ref. 9; D. Hillis, personal communication). Thus, under certain conditions, reconstructed sequences are likely to be correct.

Without a time machine we can never be certain that any given reconstructed sequence was actually present in the genome of an extinct organism. But we can ask if the recreated molecules are reasonable ancestors from what is known about their modern homologues. This is what Benner and colleagues have done for artiodactyl ribonucleases^{3,5}. Ribonuclease is expressed at high levels in the exocrine pancreas of foregut-fermenting animals such as the artiodactyl ruminants, where it is believed to serve as a digestive enzyme on RNA from foregut bacteria⁵. Early in ruminant evolution, the gene encoding pancreatic ribonuclease duplicated, giving rise to at least two other

genes that are known to be expressed in cattle, one in brain and the other in seminal vesicles⁸. Ribonuclease protein sequences are known from about 40 mammals, including many artiodactyls⁸. This high density of sequence information allowed reconstruction of ribonucleases, with few ambiguities, representing artiodactyl ancestors going back



Proper reconstruction of ancestral sequences involves inference of the most likely pathway or pathways of evolutionary events (such as amino-acid replacements or nucleotide substitutions) upon a correctly rooted evolutionary tree^{1,6,7}. Illustrated here are two of the nine possible rooted versions of the shortest bifurcating tree for the six hypothetical modern nucleotide sequences shown (U–Z). The ancestors on these rooted trees are labelled 1–5, going backwards in time. The reconstructed sequences (inferred by parsimony^{6,7}) for each ancestor on the two rooted trees are shown (question marks indicate ambiguities). Note that inferred ancestral sequences are dependent on tree topology (for example, the most ancient hypothetical ancestors, 5A and 5B, could differ at all four positions). Thus, the correct phylogeny is the foundation for correct ancestral reconstructions. In contrast, there is a singular consensus sequence (a common output of alignment programs) of U–Z, which matches neither 5A nor 5B. Logically, a consensus sequence is not the same thing as an ancestral sequence, and may only approximate to one under certain evolutionary models (see text) or through luck.

some 40–50 million years to when foregut fermentation evolved in the early ruminants^{3,5,8}.

Benner and colleagues^{3,5} created genes encoding these inferred protein sequences by site-directed mutagenesis, and then expressed the mutants in bacterial cells and studied the properties of these 'ancestral' ribonucleases *in vitro*.

All of the reconstructed ribonucleases going back to the common ancestor of the advanced ruminants (including cattle, deer, antelope and giraffe) displayed thermostabilities and kinetic properties quite similar to those of cow pancreatic ribonuclease. So parsimony inferred ancestral sequences that appear to be fully functional and stable ribonucleases^{3,5}, suggesting that the reconstructions are highly accurate. Unexpected properties were found for the reconstructed ribonucleases corresponding to the more ancient artiodactyl ancestors, including those of the pig and camel lineages. These ancient ancestral ribonucleases were slightly less thermostable than is cow pancreatic ribonuclease, and had about five times as much activity upon double-stranded RNA; a similar substrate specificity is seen for seminal ribonuclease⁵. Further site-specific mutagenesis identified a single amino-acid replacement (Gly 38 → Asp) as being primarily responsible for this shift in substrate specificity: the mutants with Gly 38 showed about five times greater activity on double-stranded RNA than did those with Asp 38 (ref. 5).

Interestingly, the gene duplications that gave rise to the brain and seminal ribonucleases probably occurred after the divergence of the lineage leading to the advanced ruminants from the lineage leading to the camels⁸. Parsimonious reconstruction of amino-acid 38 upon several feasible artiodactyl ribonuclease phylogenies suggested that a Gly 38 → Asp replacement occurred along the lineage leading to the advanced ruminant pancreatic ribonucleases after its divergence from seminal ribonuclease (which retains Gly 38)⁵. This highlights the possibility that duplication of the ribonuclease gene in the ruminants allowed tissue-specific expression and subsequent specialization of the enzymes, as is seen for ruminant lysozymes¹⁰. If so, the 'pancreatic' ribonuclease gene might have been expressed in diverse tissues in the ruminant ancestor, where it had a generalist function. Perhaps this ancestral condition is still found in pigs and camels, whose

expression and subsequent specialization of the enzymes, as is seen for ruminant lysozymes¹⁰. If so, the 'pancreatic' ribonuclease gene might have been expressed in diverse tissues in the ruminant ancestor, where it had a generalist function. Perhaps this ancestral condition is still found in pigs and camels, whose

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pancreatic ribonucleases retain Gly 38.

In a different study published last year⁴, an active ancestral molecule was deduced from a consensus sequence of F-type L1 retrotransposons that are dispersed in the mouse genome. These modern L1 sequences are transcriptionally and transcriptionally inactive, apparently because of the accumulation of mutations, but the recreated F-type monomer had transcriptional activity⁴. Although in most instances the consensus sequence is not a valid ancestral sequence (see figure), this approach appeared to work for L1. This is probably because the L1 sequences integrated into the mouse genome within the last few million years⁴, so their evolution-

ary history may be better represented by a starburst than by a strictly bifurcating tree.

To date, the cases where ancestral sequence reconstruction seems to have worked involve sequences that are fairly closely related. As sequences become available from a greater variety of species, we should be able to reconstruct ancestral molecules going further back in time: perhaps — in the future — to the very root of the 'tree of life'. □

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CATALYSIS

Electron transfer branches out

Allen J. Bard

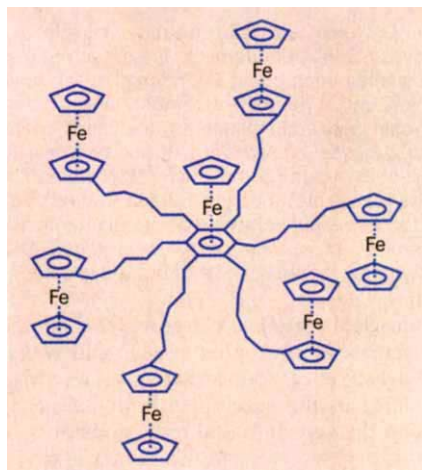
THE production of molecules that can act as multielectron catalysts is a challenge for organic chemists, but it is a goal that remains largely unrealized. Nevertheless, ingenious syntheses of new molecules¹⁻³ that show multielectron redox reactions could be bringing the goal closer.

In work published in December¹, Astruc and co-workers describe the synthesis of an interesting polynuclear iron-containing species which has six ferrocene units (FeCp_2 , where Cp is cyclopentadiene) attached to the benzene (Bz) of a BzFeCp core (see figure). This 'molecular tree' can serve as an electron reservoir, where each FeCp_2 unit can be oxidized to FeCp_2^+ , to provide an overall six-electron (6e) reaction. Cyclic voltammetric measurements in *N,N*-dimethylformamide show that the individual FeCp_2 units out on the branches do not interact appreciably with one another or with the central core, and thus are oxidized at a potential near that of free FeCp_2 in a single 6e wave. The BzFeCp core, in contrast, is oxidized at fairly negative potentials and its 1e wave serves as a convenient internal standard. The authors suggest possible applications of this substance in multielectron redox (electron transfer) catalysis and molecular electronic devices.

This molecule is only one of many that show multielectron redox reactions — examples are linear polymers, supra-molecular species and dendrimers. The latter consist of large molecules with units linked to form star-shaped or hyper-branched patterns; Astruc and colleagues' molecule could be thought of as the core of such a dendrimer. Electroactive groups can be incorporated within or on the periphery of the molecule to provide multiple redox centres. Such molecules

differ structurally from the more familiar (and easier to synthesize) linear polymers and oligomers with pendant redox groups.

One such supermolecule is a decanuclear ruthenium polypyridine complex⁴ containing linked $\text{Ru}(\text{bpy})_3^{2+}$ -like units (bpy is 2,2'-bipyridine). Molecules such as these are of interest because the photochemical and electrochemical processes of



$\text{Ru}(\text{bpy})_3^{2+}$ have potential applications in energy conversion and chemiluminescent analytical methods.

Very different dendrimeric molecules have been synthesized by Miller and co-workers². In these species, redox-active imide (A) groups were attached around the periphery of the molecule. Depending upon the size of the dendrimer it was possible to incorporate from 6 to 192 A groups per molecule and reduce these to the radical anions (A^-). In these molecules, as opposed to the molecule of Astruc and co-workers¹, the cyclic voltammetry suggests interactions among the electroactive groups, ascribed by the authors to possible π -stacking of the radical

anions. Electrochemical investigations are useful in determining the extent of interactions between electroactive groups and whether a molecule with *n* identical electroactive centres undergoes electron transfer in a single *n*-electron wave, as is typical for linear polymers with pendant groups, or in closely spaced waves at different potentials⁵.

The real pay-off with such molecules will come with the synthesis of species that can act as multielectron catalysts for useful redox reactions. Such catalysts have long been sought for the efficient reduction of dioxygen to water or dinitrogen to ammonia under ambient conditions in ways that mimic biological reductions catalysed by enzymes. For example, an effective electrocatalyst for O_2 reduction that allows the reaction to occur at potentials near that of the $\text{O}_2/\text{H}_2\text{O}$ couple would find application in fuel cells for the direct conversion of fuels to electricity. Present-day fuel cells use expensive platinum-based electrocatalysts for this function. Similarly, a good N_2 catalyst would be useful in the production of fertilizer under less extreme conditions than those that are currently employed.

But these reactions, and indeed most interesting multielectron reactions, are complicated. For example, reduction of O_2 to H_2O requires not only the addition of four electrons, but also addition of four protons and cleavage of the O–O bond. A catalyst for this process will require a more complex structure than the type of molecules described above; it will have to provide not only the needed four electrons, but also sites that bind O_2 and its reduction intermediates. Simple multielectron transfer catalysts do not work well. There has been some success with metal porphyrin-based materials as oxygen reduction catalysts — for example, last year's report³ of a Co porphyrin with three pendant $\text{Ru}(\text{NH}_3)_5^{2+}$ groups that shows electrocatalytic behaviour for O_2 reduction — but not, as yet, anything to rival platinum.

In the real world, of course, the cost of such catalysts is an important factor. Many of the more exotic molecules, such as those discussed here, are difficult to synthesize and thus expensive. At present, indeed, they often cost more than the noble metal catalysts they hope to replace. □

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Give in the filaments

Malcolm Irving

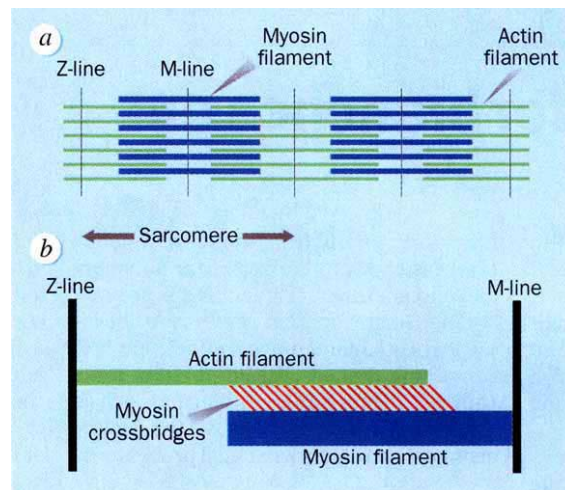
MUSCLE research has been transformed by the advent of techniques that measure the mechanical properties of single molecules, the latest development¹ being measurement of the compliance of isolated actin filaments. This corresponds to a filament extension of only about 0.2% under the force of muscle contraction. Coincidentally, advances in synchrotron X-ray technology have now allowed this tiny extension to be measured in intact muscles^{2,3}. These new results show that filament compliance accounts for at least half of the compliance of the muscle sarcomere, and challenge some of the current ideas about how muscles work.

The measurement of such small extensions is in itself a remarkable feat. Kojima *et al.*¹ used single actin filaments attached to the tip of a flexible glass needle of known compliance (that is, extension per unit force). They applied sinusoidal 10-nanometre displacements to the base of the needle, and used the deflections of the tip to determine the compliance of the actin filament plus that of the end connections. The latter was eliminated by making the measurement on filaments of different lengths. Filaments of pure actin were slightly more compliant than those containing tropomyosin, the long α -helical protein that runs along the actin filaments in muscle. Kojima *et al.* calculate that the 1.0-micrometre-long actin/tropomyosin filaments in muscle have a compliance of 0.015 nm per piconewton (pN), and would be stretched by 0.23% during isometric contraction.

Huxley *et al.*² and Wakabayashi *et al.*³ used a structural method, X-ray diffraction, to show that the actin-containing filaments are stretched by just this amount when intact muscles are activated. The muscle X-ray pattern contains an axial reflection from the 2.7-nm repeat of the actin monomers along the filament, and two prominent off-axial reflections with spacings of about 5.1 and 5.9 nm from the helical structure of the filament. The positions of these reflections were measured with remarkable accuracy (to better than 0.05%) by illuminating the muscles with intense and highly collimated synchrotron radiation, and recording the diffraction patterns on a storage phosphor imaging plate. Both groups found that the spacing of the actin-based reflections increased by 0.2–0.3% on stimulation of the muscles, in agreement with the filament extension

predicted by the *in vitro* measurements¹.

The spacings of the myosin-based reflections in the X-ray pattern increase by more than 1% when muscles are activated⁴. However, this relatively large change in spacing is not related to the force in the muscle; rather, it is due to a structural rearrangement accompanying muscle activation^{2–4}. The elastic component of changes in filament spacing can be isolated by slowly stretching an active



Structure and force generation in striated muscle. *a*, Interdigitating myosin and actin filaments are organized into repeating sarcomeres each about 2 μ m long. *b*, The fundamental mechanical unit is the half-sarcomere, in which the overlapping myosin and actin filaments are linked by the force-generating myosin crossbridges. It had been assumed that nearly all of the overall compliance ('extensibility') of muscle lies in the myosin crossbridges. But the studies^{1–3} discussed here show that is not so, and that the filaments account for 50% or more of muscle compliance. Ideas about force generation may therefore have to be re-evaluated.

muscle, to produce a large and maintained increase in force. Huxley *et al.* and Wakabayashi *et al.* found that such stretching increases the spacing of both the actin- and the myosin-based reflections by 0.2–0.3% when scaled to the isometric force. The results confirm that the increase in actin spacing comes from filament compliance, and suggest that the myosin filaments undergo a similar extension.

These filament compliances are small, but so is the compliance of active muscle. Only 4 nm of relative sliding between myosin and actin filaments is required to reduce the isometric force of muscle to zero⁵. A large contribution from filament compliance was not detected in previous mechanical measurements, and nearly all of the 4 nm was thought to be accommodated within the myosin crossbridges^{6,7}. The measurements of filament compliance^{1–3} predict that at least half of the 4 nm would be absorbed by shortening of the filaments. High-resolution

measurements of the compliance of de-membrated muscle fibres indicate that actin filaments contribute about half of the total, in agreement with this prediction (H. Higuchi, T. Yanagida and Y. E. Goldman, personal communication). The stiffness of muscle has been widely used as a measure of the fraction of crossbridges attached to actin, on the assumption that nearly all the muscle compliance was in the crossbridges⁷. The interpretation of those experiments will now need to be reconsidered⁸.

Compliance is fundamental to force generation⁶. The myosin head is thought to undergo a conformational change —

the working stroke — which produces a displacement of 5–10 nm at its tip and generates force by stretching compliant structures in series with the motor domain^{6,9}. Compliance within the myosin crossbridges themselves allows them to operate as independent force generators⁶. Compliance in the filaments could play a similar role in force generation if the working strokes were roughly synchronous, as may be the case during the rapid force recovery following a quick release of active muscle^{5,6}. In a normal contraction, however, a few hundred heads interact asynchronously with each actin filament. The filament would be effectively inextensible during the working stroke of an individual head, and a displacement of less than 2 nm would be available to each head in its local compliance as it generates the isometric force. So the local spring in the crossbridge is less than half as compliant as previously assumed.

Independent evidence that the compliance of the crossbridges may have been overestimated comes from other mechanical measurements on single molecules. Assuming that most of the myosin heads bear force in muscle, the force per head has generally been estimated as about 2 pN, giving a crossbridge compliance of 4 nm per 2 pN before filament compliance has been taken into account.

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But *in vitro* estimates^{10,11} of the force per myosin head give a value of 5 pN or more. These results mean that, after allowing for filament compliance, the compliance of the crossbridge spring may be less than 2 nm per 5 pN. As the spring compliance is reduced, so is the distance it can be stretched by thermal energy, which is a key parameter in conventional cross-

bridge theories of muscle contraction. The new mechanical and structural nanotechnologies may soon provide a definitive test of these theories. □

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GRANULAR FLOW

Mixed predictions

R. P. Behringer

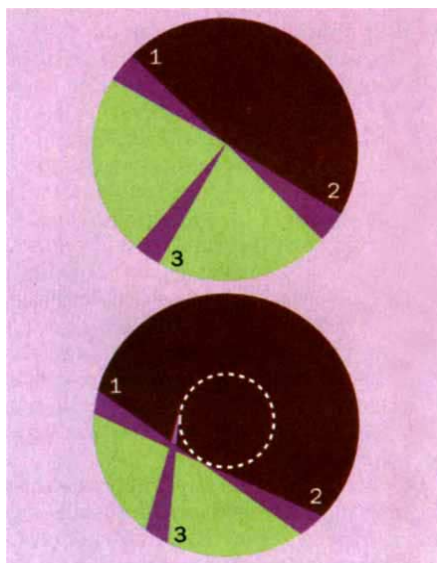
AN understanding of the dynamics of granular materials is extremely important for a host of technical processes ranging from the seemingly mundane storage and retrieval of coal in silos to the high-tech processing of pharmaceutical powders¹. As yet, our understanding sadly lags that for other materials^{2,3}. But on page 39 of this issue, Metcalfe *et al.*⁴ demonstrate that a simple but original model can describe a good deal about the particularly important process of mixing.

Granular materials can be thought of as an intermediate phase of matter: they can sustain shear like a solid — up to a point — but they can also flow like water. Once flow begins, the motion can contain a large random component, which is sometimes described in terms of a granular temperature. Yet energy, random or otherwise, is rapidly dissipated for granular flows. And, in many cases, static regions adjoin flowing regions. A conventional thermodynamic or hydrodynamic description is not appropriate. So what Metcalfe *et al.* have done is to combine experiments with a simple but novel model for mixing of a homogeneous material. By contrast, much other work has focused on the segregation that occurs for mixtures of particles of different sizes^{5–7}. A key feature of the work by Metcalfe *et al.* is that it cleanly separates by deterministic and random aspects of mixing. From this work comes a specific prediction for optimum mixing efficiency.

Metcalfe *et al.* consider an idealized drum mixer which is thin enough that the motion is effectively two-dimensional. The axis of the drum is horizontal, and as it rotates, the upper surface of the material inside becomes unstable to avalanches. The rotation rate is slow, so that each avalanche stops before the next begins. The instability angle is θ_i , and after an avalanche, the surface of the material relaxes to one with a lower slope, $\theta_f < \theta_i$. The effect of an avalanche is to transfer a thin wedge of material from the upper to the lower part of the surface. Once material has fallen to the lower side of the incline, it remains there until rotation of the drum brings the

material back to the top once more.

Metcalfe *et al.* find experimentally a very complex process as the grains avalanche down the upper surface, and an exactly describable rotation thereafter, until the material once again is ready to avalanche. If the drum is exactly half full, a wedge will avalanche and rotate repeatedly without any mixing between wedges. But by simple geometry, if the drum is less than half full, a wedge of material from a given avalanche will not stay in the same wedge on successive avalanches: different wedges will mix. This is a purely geometric process, as sketched in the figure. In each case, position 1 corresponds to the point at which a wedge avalanches. After the avalanche, the wedge is at position 2. Position 3 corresponds to a later time when the wedge has rotated part way around. For the first case, nothing will have happened to the wedge in question. But for the second case, the tip of the wedge will already have been mixed with other material at point 3. Mixing between wedges also occurs if the drum is more than half full, so that $f = 1/2$ is a particularly inefficient filling fraction for mixing. If



Mixing 'wedges' of granular material. *Top*, for a half-filled drum of grains; *bottom*, for a drum that is less than half full.

one characterizes the mixing by γ , the rate at which material within the container becomes homogenized, this rate is largest for small filling fraction f ; falls to zero (no mixing) for $f = 1/2$; rises somewhat for f increasing above $1/2$ and is again zero for a full container, $f = 1$.

The details of how mixing occurs during an avalanche are unclear. In their model, Metcalfe *et al.* assume that this is a random process. They then incorporate the geometric effect of wedges as described above, which is totally deterministic.

Although γ is an important measure of mixing, a practical concern is to produce as large a volume of mixed material as possible in a given time. If V represents the fractional volume of the drum which is occupied by material, then one measure of the efficiency is $\chi = \gamma V$. Metcalfe *et al.* predict a maximum for χ when $f = 0.25$, and they obtain a maximum efficiency in their experiments when $f = 0.23$. This is excellent agreement.

Important questions remain. Perhaps the most interesting and technically relevant of these is what happens if the grains have a range of sizes. This work shows that the important additional feature to describe this process will be the dynamics during the avalanche. For instance, if one size of particle consistently tends to avalanche closer to the curved wall of the drum, there will be radial segregation by size, as in the recent experiments of Clément *et al.*⁸. In order to capture such features, the model must account for each grain size separately. In the simplest case of two distinct grain sizes, the geometric part of the model would clearly need modification to account for the fact that, after the avalanche, the particles of a given size would no longer be distributed uniformly in the wedge. But this avalanche-induced segregation is unlikely to be perfect, and thus the random part of the model would also need to be modified. Additional issues that will need to be addressed include three-dimensional effects, and mixing in non-cylindrical geometries. Although the mathematical analysis may be more complicated in these cases, the key idea of separating geometric and random mixing will still apply. □

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The duck's dilemma

Peter Parham

THE Y-shaped antibody is a quintessentially bifunctional protein: unique binding sites of the arms latch on to intruders, while 'effector' sites in the stem ensure delivery of such undesirables to the destructive forces of complement and phagocytes. In the laboratory, proteases are used to cleave arms from the stem and separate the two functions, but such tricks contribute little to the quotidian working of the mammalian immune system.

Not so for ducks and other waterfowl. As their immune response develops, bifunctional antibodies give way to a smaller species which binds antigen but cannot summon the effectors. This unusual progression has been watched for years. But until publication of papers by Gregory Warr and co-workers in the *Journal of Immunology*^{1,2}, the latest of which appeared in December last year, its mechanism remained a tease. Magor *et al.*^{1,2} find alternative splicing of messenger RNA to be the culprit. Although we mammals use the strategy to remove membrane anchors and 'convert' the receptors of resting B cells into secreted antibodies, the ducks take it a stage further by trimming off two domains of the constant region.

In the balmy days before mice became the *Escherichia coli* of immunology, it was not uncommon for immunologists to work with other animals, usually those found on farms. Duck antibodies raised against kidney were a favourite for the induction of experimental glomerulonephritis in rats. Amongst others, Emil Unanue^{3,4} and Howard Grey⁵⁻⁷ spent the 1960s studying the idiosyncracies of duck immunoglobulins and their apparent deficiency in effector functions. As in other species, the first serum antibody produced in the duck's response is IgM, which is then superseded by IgY. This predominant immunoglobulin of avian serum was for some years considered the equivalent of mammalian IgG, but evidence against this 'mammo-centric' view has been building^{1,2,8,9}. Indeed the textbook claim that the most widespread serum antibody is IgG may be a canard, for the appropriately named IgY is the major constituent antibody of serum in birds, reptiles and amphibians.

Ducks, geese and swans are distinguished from the chicken (the avian immunologist's first love¹⁰) and other birds by the manufacture of a small version of

IgY, which emerges later in the immune response than the full-sized model but then becomes the dominant species. From comparison of the protein isoforms, Grey⁵ showed that the large and small versions of IgY differ in the size of their heavy chains. From comparison of complementary DNA and genomic clones, Magor *et al.* now explain precisely how that comes about.

To favour secretion of the large isoform of IgY, Magor *et al.* immunized one group of White Pekin ducks using a light regime

proteolytic interconversion or other post-translational effects. Two viable models remained: alternative mRNA splicing of a single gene or the transcription of different genes (or sets of genes). Given the many surprises lurking in the immunoglobulin genes of species exotic to the mainstream immunology laboratory^{10,11}, both were tenable.

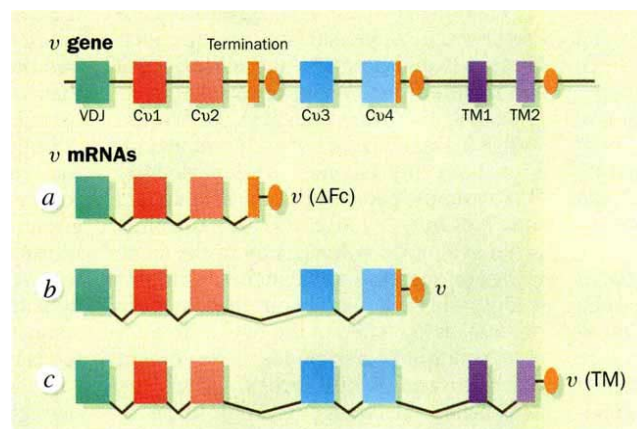
To distinguish between them, Magor *et al.* examined the organization of the *v*-chain constant-region domains within the duck genome and found but a single gene². Moreover, within the intron between exons encoding the second and third constant-region domains was a small 'terminal' exon encoding the two carboxy-terminal amino acids of the truncated

form (see figure). This pointed to a mechanism of alternative mRNA splicing, which was then demonstrated by showing that the precise splicing of sequences predicted from this arrangement actually occurs. So the smaller, more mature isoform of IgY is an antibody without the domains corresponding to the Fc (crystallizing fragment) region of mammalian IgG. Magor *et al.* thus designate this species as IgY(Δ Fc) and the heavy chain as *v*(Δ Fc). In IgG, Fc is the seat of effector function. Its absence from most antibodies of duck serum may make for wonderful soup, but how does it help the duck?

The molecular biology is standard fare, straight from the menu of chef Maniatis. But the

immunology is bizarre. Why, right in the middle of getting an immune response going, does the duck play around with the bifunctionality of its antibodies? Immunoglobulin without Fc is a defender under severe restraint — it may obstruct, bully, inhibit and cajole but it cannot dispatch. Underlying this chickening out through alternative mRNA splicing there must be a dilemma, some sort of immunological catch-22.

Warr and co-workers believe that the explanation lies with structural similarities indicating that IgY is the common ancestor of IgG and IgE, and evidence that it combines properties of each of these two classes of exclusively mammalian antibodies⁹. IgG is abundant and binds diverse antigens in ways that are demonstrably helpful. IgE, on the other hand, is mostly a nuisance which can start with inducing hay fever and work its way up to asthma and anaphylactic shock. Luckily IgE is made in minute amounts, but this boon is offset by the extraordinarily high affinity of its constant-region domains for the IgE receptor on the histamine-producing mast cells. If, as Magor *et al.*



Exon-intron organization of the constant region of the *v* heavy-chain gene in White Pekin ducks. Alternative splicing of messenger RNA produces, *a*, the form lacking an Fc region (IgY(Δ Fc)); *b*, the full-sized, secreted IgY; and *c*, the membrane-bound 'receptor' form. TM, transmembrane domains; cleavage/polyadenylation signal is in orange. (Adapted from Fig. 1 of ref. 2.)

of intramuscular injection over a period of 11 days. In contrast, to enrich secretion of the smaller isoform other ducks were immunized, repeatedly and intravenously, over 70 days¹. Afterwards the ducks' spleens were used to make libraries from which cDNA clones encoding IgY heavy chains — the epsilon *v*-chain — were isolated. Nucleotide sequences revealed that the larger chain consists of a variable-region domain (V_v) and four constant-region domains (C_v), whereas the smaller *v*-chain has just the variable and amino-terminal two constant-region domains. Replacing the two carboxy-terminal constant-region domains in the shorter *v*-chain are two amino acids, glutamate and phenylalanine, the second of which corresponds to the carboxy-terminal amino acid determined by classical protein chemistry⁵.

Implicit from the cDNA analysis is that the long and short *v*-chains are encoded by discrete species of mRNA, as Magor *et al.* were then able to demonstrate directly¹. Their findings ruled out previously considered models of the relationship between the two forms of IgY based upon

suggest, avian IgY combines the *yin* of IgG with the *yang* of IgE, then ever close on the heels of its protection against infection is the risk of an anaphylactic demise. By the trick of alternative splicing, ducks can raise the concentration of antigen-binding sites without a commensurate increase in the engagement of potent effectors.

Managing an immune system is fraught with compromise. Whether ducks and waterfowl have come off worse than chickens and the rest of us remains to be seen. Given the gentle nature of their immunoglobulins, it may not be coincidence that ducks maintain a more friendly relationship with viruses than we do⁹. For example, much attention has been paid to influenza, which is fully adapted to waterfowl but causes them no disease¹². Ducks seem to act as worldwide reservoirs for avian influenza viruses which are hypothesized to contribute to new pandemic strains of human influenza virus, a genetic mixing that may be facilitated by the co-location of pigs, humans and Pekin ducks in southern China^{12,13}. It may there-

fore be helpful to understand more of how these species cope with the viruses, and to gain guidance from Donald as well as from Mickey. □

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PLATE TECTONICS

The rise and fall of Tibet

Mike Searle

The Tibetan plateau is the largest uplifted part of the Earth today, occupying an area of some two million square kilometres at an average elevation of just over 5,000 metres. Nevertheless, we are not seeing it at its peak; at some point since its uplift, the plateau has begun to collapse. Now Coleman and Hodges, on page 49 of this issue¹, have set a date of 14 million years ago on the start of that collapse, and thus a minimum age on the rise of the plateau. The timing of uplift, maximum elevation and subsequent collapse of the plateau is a question of great interest. Not only is it needed for understanding tectonic processes and the mechanism of forming thick continental crust, but it also features prominently in global atmospheric circulation models of past climate.

Whereas most of the plateau is remarkably flat, it is surrounded by some of the highest, most deeply eroded mountain ranges on the Earth. To the south lies the Himalaya; to the southwest, the Karakoram; north, the Kun Lun–Altyn Shan–Qilian Shan range; and east, the Longmen Shan. There seems little doubt from the position of the plateau, immediately north of the Himalaya, that the unusual crustal thickness under Tibet (at 50–70 km thick, twice the normal value) was a direct consequence of the collision of India with Asia². The timing of the India–Asia collision is reasonably well constrained at 54–49 Myr ago — between the uppermost

Palaeocene and Middle Eocene — from the dating of the last marine sediments on the Indian plate margin and within the Indus suture zone^{3,4}. Since the collision, folding and thrusting have shortened Indian plate crust of the Himalaya and

south Tibet by hundreds of kilometres. North of the Indus suture zone, the Asian plate crust of the Tibetan plateau has been undergoing homogeneous deformation involving north–south shortening, resulting in the double crustal thickness and 5-km elevation. But fault plane solutions of earthquakes and the active tectonics of the plateau show that, although India is still moving northwards relative to stable Siberia at a rate of 4–5 cm per year, normal faulting and east–west extension are dominant within most of Tibet today, whereas thrust faulting and strike-slip faulting are dominant in the mountain ranges bordering the plateau⁵. Since the India–Asia collision, the Tibetan plateau has been growing in elevation and crustal thickness. At some time in the past, the plateau exceeded its maximum sustainable elevation and began to collapse. The evidence is a system of north–south normal faults indicating east–west extension (Fig. 1)⁶. If we can date the movement on these normal faults, it should therefore give us a minimum age on the maximum elevation of the plateau.

That is what Coleman and Hodges¹ have done. They present ⁴⁰Ar/³⁹Ar age data from hydrothermal muscovites growing along a north–south aligned fault associated with one of the largest graben systems, the Thakkola graben (Fig. 2). These faults have acted as channels for fluid flow along which hydrothermal minerals have grown. Their age of about 14 Myr provides, for the first time, a lower boundary to the onset of east–west extension along the southern part of the plateau, and it is considerably older than previously thought. The north–south

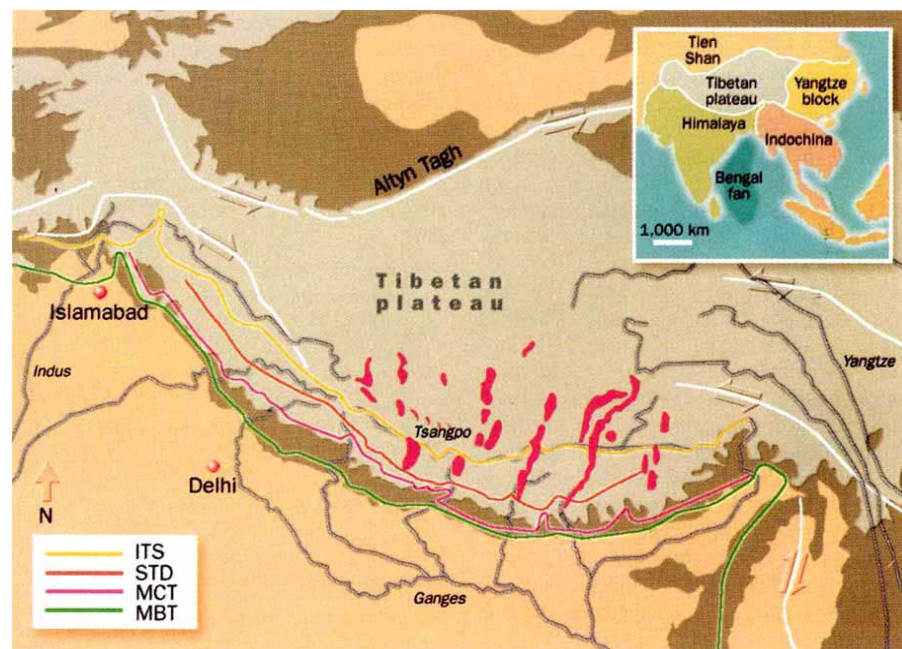


FIG. 1 Simplified map of Tibet and the Himalaya showing the extent of the north–south graben systems (shown in red). ITS, Indus Tsangpo suture; STD, South Tibetan detachment; MCT, Main Central Thrust; MBT, Main Boundary Thrust. Area coloured grey has average elevation of 4–5 km. (Adapted from ref. 8.)

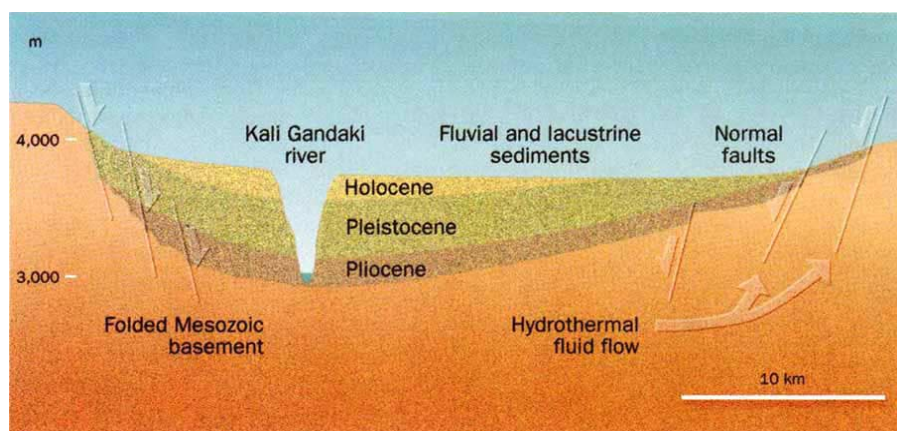


FIG 2. Cross-section of the Thakkola graben. (Adapted from ref. 14.)

aligned normal faults cut the east–west aligned mylonite fabric associated with the South Tibetan detachment, the normal fault bounding the top of the High Himalayan slab. Muscovites from the mylonites, dated¹ at 17.6 ± 0.3 Myr, provide a minimum age constraint on exhumation of the High Himalaya in the Annapurna area. Between 17.6 and about 14 Myr, therefore, the stress field in southern Tibet changed from north–south compression to east–west extension.

Why should a single date be so exciting? The only previous age reported from a normal fault in Tibet is the $^{40}\text{Ar}/^{39}\text{Ar}$ cooling age of 8 ± 3 Myr from the Nyainqentanglha metamorphic core complex^{7,8}, a completely atypical structure in central Tibet that is aligned northeast–southwest. This has been used to argue that the collapse of the plateau was contemporaneous with marked climate change in south Asia and strengthening of the Indian monsoon at about 8 Myr (ref. 9). Coleman and Hodges' new age proves that the plateau must have been high long before the apparent main changes in vegetation and climate occurred. This older age for the maximum height attained by the plateau is supported by $^{40}\text{Ar}/^{39}\text{Ar}$ ages of about 13 Myr from potassic basalts and minor felsic magmas¹⁰ in Tibet, which imply melting of the lithosphere.

Nevertheless, it seems likely that the uplift of a feature as large and as high as the Tibetan plateau and the 2,500-km-long Himalayan mountains would have had a profound effect on global circulation, climate and erosion. The uplift of Tibet would have disrupted the west-to-east air flow across the Northern Hemisphere, initiated the monsoon-driven wind systems across India and increased the precipitation along the Himalaya, with higher chemical erosion rates causing a drawdown of atmospheric carbon dioxide and resulting in global cooling^{11,12}. The rise of Tibet probably coincided with the initiation of the Indian Ocean monsoon, although the Himalaya today provides the most spectacular and abrupt northern

barrier to the monsoon. The maximum rates of erosion and exhumation along the High Himalaya in India, Nepal and Bhutan probably occurred between 21 and 19 Myr ago when the main bounding structures — the Main Central Thrust at the base, and the South Tibetan detachment normal fault at the top of the slab — were active, and most of the tourmaline-bearing leucogranites were being exhumed¹³.

The surge in geological data from the Himalaya in the past 15 years has resulted in a spectacular leap in our understanding of collision-related processes. Tibet, however, remains an enigma. Considering the huge size of the plateau, our knowledge of its geological secrets is still based on few facts and a lot of speculation. Crucial factors such as the elevation history of the plateau may be unravelled by a detailed and wide-ranging palaeobotanical study. But the timing of the switch from north–south compression to east–west extension will only be unravelled by mapping in detail all the graben systems in Tibet and, following the lead given by Coleman and Hodges, dating the faults. □

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DAEDALUS

Shaking down

LAST week Daedalus identified the human stomach as a low-frequency motion-sensor. It detects our bodily movements for feedback control purposes. We sense its action in a suddenly-dropping lift, and suffer from its misinterpretations in motion sickness.

Daedalus is now extending this bold theory. Could the 'morning sickness' of pregnancy be a form of internally triggered motion sickness due to the movements of the unborn child? Still more intriguing, could our gastric motion-sensor be the long-sought weight-stat that governs the deposition of body fat? Millions of despairing dieters know how strongly the weight-stat resists all attempts to override it downwards. Many people maintain the same weight for years, no matter what they eat; the body clearly knows its own weight rather well. Daedalus now suggests that it monitors not weight but inertial mass, and that its sensor, appropriately enough, is the stomach. Any change in the characteristic motion of the body is sensed directly by the stomach, which reacts by altering the efficiency with which it digests incoming food. This explains why the belly is such an important site for fat deposition. It provides good mechanical feedback to the stomach beneath.

This theory offers some comfort to those naive joggers and exercisers who believe that fat can be dissolved by jiggling it up and down. At least they are providing their gastric motion-sensor with clear signals: a perfectly sedentary fatty would leave it guessing. DREADCO volunteers are now being dotted with small accelerometers, and loaded up with carefully weighed slabs of artificial fat. They are then being put through a variety of exercises, to discover how the added mass affects their main bodily frequencies, their digestive efficiency and their weight.

Slimmers everywhere will await the results with keen interest. If a proper distribution of weights can indeed fool the weight-stat into overestimating the body mass, a new fat-reduction technology will be born. DREADCO will market special slimming undergarments padded out with a nicely judged distribution of plastic 'fat'. The happy wearer will lose weight without dieting or willpower — although his or her new elegant figure may be camouflaged beneath the cumbersome garments themselves. A well designed programme of low-frequency vibro-massage, repeatedly subjecting the body to the correct vibrational pattern, might do a neater job. Sadly, it might give the user severe motion sickness. David Jones

Visual fields in ostriches

SIR — Vertebrate eyes differ markedly in size and position in the skull. Although general principles accounting for their diverse optical designs have been identified^{1–3}, general explanations of the form and function of their associated visual fields, especially binocular fields, have been elusive^{3–5}. Here we show that in ostriches (*Struthio camelus*), whose eyes are among the largest of all land vertebrates and the most widely separated of all birds, the binocular field is surprisingly small. We also show that similarly narrow frontal binocular fields occur in other birds which differ markedly in their eye size and eye separation and also in their ecology and phylogeny. In common with these species, ostriches use visual control of bill position during highly accurate pecks while foraging for food. Species that do not use their bill in this way have different binocular field topographies. We propose that in birds, foraging behaviour is the principal determinant of binocular field characteristics.

We investigated typical head positions and feeding behaviour in ostriches by taking photographs and video sequences of individuals feeding on grains and alfalfa leaves. When stationary, walking, raising or lowering its head, an ostrich holds its bill in an approximately horizontal plane (see figure). Usually, the head is tipped downwards only before feeding. Despite their large heads and bill size, ostriches feed using highly accurate and rapid pecking; individual items are taken in single pecks which occur in the direction of the plane of the bill. This pecking seems to be entirely under visual control. As in other birds^{6,7}, the peck shows a relatively slow approach and/or a static phase, followed by a rapid ballistic movement during which the bill begins to open and the eyelids or nictitating membranes start to close. The ballistic phase begins when the bill tip is 1 – 1½ bill lengths from the target, 75 – 110 mm from the bill tip. Video sequences showed no

evidence of large-amplitude eye movements, although these cannot be discounted entirely.

Visual fields were measured using an ophthalmoscopic reflex technique⁸. The binocular retinal field is small. It extends vertically through 80° and has a maximum width of about 20° in a region which extends from the horizontal plane to 10°

of the bill tip. The proximal limit of this blind area coincides approximately with the position where during a peck the final ballistic phase begins. Note that even if convergent eye movements are present, the binocular field in the region of the bill would still be about 20°, as the bill itself limits the field margins. Only if the eyes were farther apart could a wider binocular field, and a smaller blind area in front of the bill tip, be achieved.

In view of the apparent prominent

RETINAL VISUAL FIELDS, AXIAL EYE LENGTH AND SEPARATION OF EYES IN 8 BIRD SPECIES

Species	Retinal field (degrees)			Eye linear dimensions (mm)	
	Binocular	Monocular	Cyclopean	Axial length	Nodal separation
Ostrich	20	155	290	39.0	82.0
Cattle egret	22	167	321	13.0	22.0
Night heron	22	171	320	17.7	29.0
Stone-curlew	18	181	345	20.5	32.0
Pigeon	22	169	316	11.6	16.0
Woodcock	4.5	182	359	16.9	20.0
Mallard	8	183	360	13.5	18.0
Tawny owl	48	124	201	29.0	40.0
Human	140	170	200	24.0	61.2

Human data are included for comparison. All visual-field data were obtained using a similar ophthalmoscopic reflex technique and refer to visual fields in an approximately horizontal plane when the eyes have adopted a relaxed position and the head is in a typical posture for the species (see refs below for details of these postures). Axial lengths of eyes and separations of their nodal points in the skull are from the cited references, except ostrich and human, which are based on personal observations. The foraging of ostriches, herons, stone-curlews and pigeons all use visual guidance of the bill. Foraging in woodcocks and mallards is guided by tactile cues from the bill, and tawny owls use auditory and visual cues to detect prey which are taken in the feet. Details of visual fields and foraging behaviour from the following references: Cattle egret (*Bubulcus ibis*), 8, 14; night heron (*Nycticorax nycticorax*), 14; stone-curlew (*Burhinus oedicnemus*), 15; pigeon (*Columba livia*), 16, 17; woodcock (*Scolopax rusticola*), 18; mallard (*Anas platyrhynchos*), 19, 20; tawny owl (*Strix aluco*), 16, 21; human, 22.

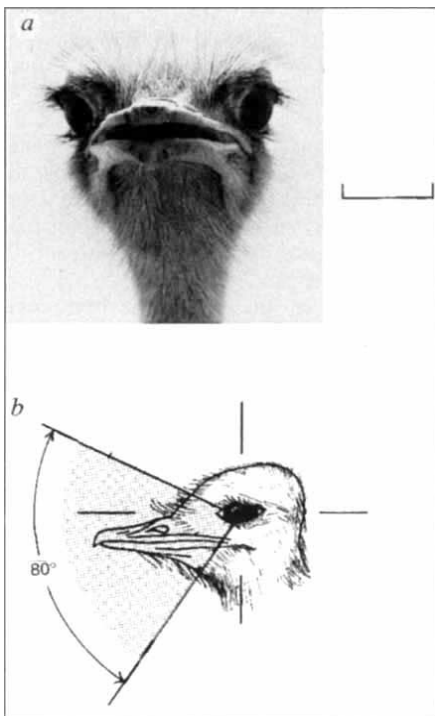
below the bill (*b* in the figure). The optic axes project forwards and downwards and, as in other birds³, the monocular fields are asymmetrical about each axis. There is a large blind area above and behind the head. In the frontal field at the elevation of the bill and below it, the margins of the retinal field coincide with the bill. Reconstruction of the field margins in relation to the skull and bill showed that although there is a 20° binocular area in front of the bill, functional binocular vision does not begin until approximately 130 mm from the bill tip — there is a blind area in front

position and wide separation of eyes in the skull (*a* in the figure), the size of the frontal binocular field appears surprisingly small. This discrepancy between appearance and the measured field arises from the asymmetry of the retinal fields about the optic axes. This gives rise to a blind margin to the optical field at the nasal limit. Thus, although both pupils can be clearly seen, giving the appearance of a large binocular field, ophthalmoscopic observation readily confirms that much of this is not served by retina.

This relatively narrow binocular field about the bill is, however, not unique. Other species using visually guided pecking for feeding have similar narrow frontal binocular fields. The table shows that such similarity occurs independently of the width of monocular and cyclopean visual fields, eye size and separation of eyes in the skull, as well as differences in phylogeny and ecology. Thus, a narrow binocular field about the bill can probably be regarded as a convergent feature of species which feed using visually guided pecking. This is supported by converse data from woodcocks, mallards and tawny owls, which forage using mainly non-visual cues (see table). In these three species the bill falls outside the visual field. In owls

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a, Head of an ostrich in a natural posture with the bill approximately horizontal. Photographed in natural midday sunlight. Scale bar, 50 mm. b, Lateral view of an ostrich head indicating the vertical extent of the region in which binocularity occurs. Maximum binocular width of 20° occurs from the horizontal to approximately 15° below the bill. Retinal field measurements were made on intact heads ($n=4$) obtained within minutes of death at an ostrich farm abattoir. All measurements completed within 30 min of death; intraocular pressure and optical quality remained high throughout the measurements.

the relatively large degree of binocular overlap in the horizontal plane can be correlated with the position of the feet as they are swung upwards and forwards to take prey.

Although the apparent convergence upon a narrow frontal binocular field size is striking, the perceptual factors with which this is correlated are not clear. It is thought that accommodation rather than stereopsis is the primary cue used to guide absolute distance perception in pecking⁹. In pigeons pecking accuracy is not diminished by monocular as opposed to binocular viewing¹⁰. Thus, binocularity *per se* may be less important than the fact that each monocular field extends contralaterally. In the pecking species of the table this equals approximately only 10° , and in the plane of the peck the width of the contralateral field will always be limited by the bill's shape and its position relative to the eyes.

Whereas the topography of the frontal visual field appears to be constrained by the nature of the foraging task, visual field topography to the rear of the head varies markedly (see refs in the table for detailed descriptions). Ostriches have a large blind

area above and to the rear of the head which is typically held so that the eyes are shaded from the sun (a in the figure). Ostriches live primarily under cloudless skies at latitudes where the sun is at high altitude throughout the day¹¹. Shading the eyes may be crucial in preventing retinal damage caused by imaging the sun. In vertebrate eyes, retinal illumination remains uniformly high for images up to an eccentricity of 70° from the optic axis¹². Thus, even a peripheral image of the tropical sun could cause damage as well as degradation of the retinal image in other parts of the ostrich's visual field¹³.

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Amazon mercury emissions

SIR — Veiga *et al.*¹ estimated inputs of mercury from forest burning in the Amazon to reach nearly 90 t yr^{-1} . They suggested that this is a major source of Hg to the region's atmosphere, which implies that the contribution of Hg from gold mining is not a significant cause of Hg contamination in fish and people of the region^{2,3}. In my view, Veiga *et al.* overestimated Hg atmospheric emissions by at least a factor of 10.

Estimating Hg atmospheric emissions from forest burning depends mostly on deforestation rates due to the large area involved, and on the forest biomass and its Hg content, and on the efficiency of Hg release during burning. Veiga *et al.*'s deforestation rate of $35,000 - 50,000 \text{ km}^2$ does not agree with estimates reviewed in refs 4 and 5, based on data from the National Institute of Space Research/National Institute of Amazon Research (INPE/INPA, Brazil) and NASA. These estimates range from $15,200 \text{ km}^2$ by NASA to $21,600 \text{ km}^2$ by INPE/INPA, with $19,900 \text{ km}^2 \text{ yr}^{-1}$ as a probable average. All studies agree that deforestation rates decreased from $19,000 \text{ km}^2$ for 1988–89; to $13,800 \text{ km}^2$ for 1989–90; to $11,100 \text{ km}^2$ for 1990–91. This deforestation rate reduces the estimate of Hg emission made by Veiga *et al.* by a factor of 3 to 5.

There is no consensus on forest biomass in the Amazon. Estimates vary by a factor of 2.0 (ref. 6). Veiga *et al.* used 351 t ha^{-1} for total biomass (standing live biomass + dead + below ground roots + fallen trunks). This is an accepted value for "dense" Amazon forest, which consists of 50% at most of the total forests in the

region^{6,7}. But non-dense forests reach 220 t ha^{-1} (34% of the area); the *cerrado*, 71 t ha^{-1} (13%); and the *savanas*, 110 t ha^{-1} (3%). I do not include *Cerrado* and *savanas* in my estimate, as they occur mostly along the Amazon border and were not considered in the rates estimated in refs 4, 5. Taking into account the distribution and biomass of the two forests units, Brown *et al.*⁷ and Martinelli *et al.*⁶ give an average total biomass of 288 and 247 t ha^{-1} . 70%–82% of that of Veiga *et al.* and resulting in an additional 30% decrease in Hg emission. I use 270 t ha^{-1} as the most likely average for forest biomass in the Amazon.

The next step to calculate Hg emission per ha is the Hg content in the biomass. Veiga *et al.* used Hg concentration in plants of $0.05 \mu\text{g g}^{-1}$ dry weight, based on inventories from temperate areas. There is no inventory of Hg content in the biomass of Amazon forests. The only data for the region given by Veiga *et al.* are from aquatic macrophytes, which have much higher concentrations of Hg than terrestrial plants. Furthermore, the data mentioned are from areas affected by gold mining⁸. Which again I believe are overestimates (see, for example, ref. 9, which gives $0.03 \mu\text{g g}^{-1}$). Additionally, Hg content in soils are also from temperate areas and typically range from 0.1 to $0.3 \mu\text{g g}^{-1}$ of soil. In the Amazon, where no Hg mineralization occur, background Hg in soils ranges from 0.02 to $0.08 \mu\text{g g}^{-1}$, even for soils with high organic matter soils^{2,3,8}. Also, 80% of the Amazon forest biomass consists of trunks, with very low Hg concentrations ($0.02 \mu\text{g per g}$). Hg concentrations in trees and soils of the Amazon are probably well below those reported for temperate regions.

Another source of error is the extremely high Hg concentration Veiga *et al.* give for "humus", which I assume is litter. Decomposing litter in tropical forests presents much lower concentrations of chemical elements than those present in temperate forest litter, mostly due to the fast nutrient recycling aided by the effi-

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ESTIMATED HG EMISSIONS FROM FOREST BURNING IN THE AMAZON

Biomass	t ha ⁻¹	Hg (µg g ⁻¹)	Hg release efficiency (%)	Hg release (g per ha)
Above ground wood and litter (roots, fallen trunks and standing trunks)	216	0.02	90	3.8
Above ground leaves and below ground roots	54	0.03	75	1.2
Soil organic matter	47	0.30	20	2.8
Total	317			7.8

cient help of mycorrhizae. Plant litter in the Amazon shows lower concentrations than the living leaves¹⁰. Therefore I will use the same concentrations used for trunks for this compartment. The soil organic matter Hg content used by Veiga *et al.* is in agreement with reported values for the Amazon, and their value for efficiency of Hg release during burning seems fair.

Recalculating the estimates of Hg release to the Amazon atmosphere through forest burning (see table) and using the deforestation rates of 1990–1991 of 11,100 km², I obtain approximately 8.7 t for this year. Even taking the largest estimates of 1978–1988, roughly 17 t of Hg would have been emitted annually during that period, still four to five times lower than the proposed estimate of Veiga *et al.* Even this value is likely to be an overestimate, as Hg concentrations in biomass from the Amazon forest as well as in soil organic matter will probably be much lower. In addition, many deforested areas are now in various stages of regrowth following abandonment, therefore actually accumulating Hg in their fast-growing bio-

mass. Thus tentative estimates of total Hg released from deforestation during the past 20 years are useless. Finally, a significant proportion of the biomass remains intact after burning, keeping a fraction of the Hg preserved as partially burnt vegetation and as coal.

Using the year 1990 for comparison, Hg emission to the atmosphere in the Brazilian Amazon due to gold mining ranged from 70 to 100 t (refs 2, 3). Recently, this type of mining spread throughout other Amazonian countries. In Venezuela, 40 to 50 t of Hg are released by this activity³. In Colombia, Peru, Ecuador and Guyana, at least 10–20 t more are released annually², and more mining fields are being opened every year. Therefore, although deforestation is a serious environmental threat to the Amazon, if there is any “significant villain” in the emission of Hg to the Amazon, it is definitely gold mining.

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Origin of chondrules explained

SIR — Chondrules generally are millimetre-sized, near-spherical stony objects that are the main constituent of the most primitive types of meteorite. Many regard chondrules as having formed in a protoplanetary disk by complete or partial melting of nebular solids. In contrast, I believe that chondrules probably resulted from interactions between already differentiated, partly molten planetary bodies.

My view was, unfortunately, misrepresented in the News and Views by Ash¹ by his statement that I “... prefer an origin in a modified, geologically undifferentiated planetary body. . .”. Since the early 1970s, when plagioclase glass with a planetary chemical signature was discovered in an ordinary chondrite², I have tried to demonstrate that some chondrules at least

were the product of differentiated planetary bodies³. Furthermore, Ash perpetuates a common misconception that chondrules have “approximately solar” “relative abundances of refractory trace elements”. At the conference on which his article was based, after exchanges between several participants, it was agreed that chondrites (the bulk meteorites) have approximately solar elemental abundances but that there is “large compositional variability of chondrules from a single meteorite, reflected in major variations of Mg/Si ratios, of Al and other refractory element abundances. . .”⁴.

The ‘magic’ to be explained is how chemically diverse objects, chondrules, be they nebular or planetary, when mixed in nature in gram-sized or larger amounts, always produce uniform, near solar compositions.

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The world's oldest songbird

SIR — The songbirds or passerines (Passeriformes) are the most speciose and widely distributed order of living birds, comprising about 60% of the 9,000–10,000 Recent species and occurring on all continents except Antarctica. It is not known whether the Passeriformes had a northern or southern origin. Traditional, pre-continental drift views held that Australo-Papuan passerines were derived from and often confamilial with Northern Hemisphere invaders¹, an interpretation challenged by palaeontological evidence and molecular studies². These latter studies concluded that the many endemic Australo-Papuan passerines were part of a large-scale autochthonous radiation from but a few ancestral types, overlaid by more recent northern invaders².

These ideas have yet to be verified by unequivocally identified fossils; all purported Eocene and Oligocene passerines before 1989 were misidentified³. The earliest undoubted passerines come from Late Oligocene deposits in France⁴. The extensive early Tertiary sites in North America and Europe contain many small arboreal forms, most belonging to the Coraciiformes; none is passerine³. It has been proposed that passerines had a southern origin, not spreading to the Northern Hemisphere until the mid-Tertiary⁵. Unfortunately, there is no evidence of early Tertiary passerines in Southern Hemisphere deposits^{6,7}. An Early Eocene site from Murgon, southeastern Queensland, has now yielded material identifiable as passerine.

The matrix yielding the fossils is green clay, radiometrically dated potassium/argon; illite) at $54.6 \pm 0.05 \times 10^6$ years⁸. This age is corroborated by non-avian elements of the Tingamarra local fauna, which includes Australia's oldest frog, trionychid turtle, madtsoiid snake, marsupials, bat, and non-volant placental mammal. The fossils of passerine and other birds form the earliest modern, non-marine avian assemblage from Australia⁹.

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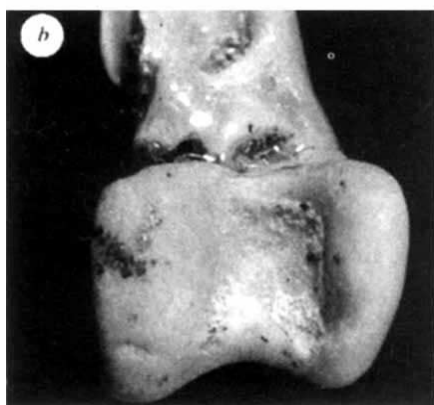
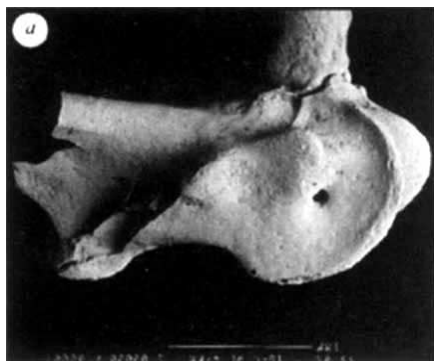
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The passerine specimens consist of a proximal right carpometacarpus (Queensland Museum F20688) and distal right tibiotarsus (QM F24685), comparable in size to a grassfinch (*Estrildidae*) and thrush (*Turdus*), respectively, indicating that they represent two taxa. Although the proximal carpometacarpal portion is fragmentary (*a* in the figure), it exhibits a large intermetacarpal process, a structure found in the Passeriformes and only a limited number of other avian orders: Galliformes, Coliiformes, Coraciiformes and Piciformes. From these the Murgon carpometacarpus is separated and identified as belonging to the Passeriformes by a combination of the shapes of the rims of the carpal trochlea, position and caudal extension of the intermetacarpal process, position of the pisiform process and the condition of the area between the extensor and pisiform processes. The tibio tarsus (*b* in the figure) is broken through the shaft; the distal portion has been repaired but is largely complete. This specimen is allocated to the Passeriformes by the condition of the lateral and medial margins of the shaft, and the shapes and positions of lateral and medial condyles, supratendinal bridge and anterior intercondylar fossa. A more detailed presentation of the diagnostic characters of the Passeriformes for these skeletal elements will appear elsewhere.

These are the oldest known passerines



Passerine remains from Early Eocene deposits at Murgon, Queensland; *a*, proximal carpometacarpal fragment (QM F20688), ventral view (line equals 1 mm); *b*, distal tibiotarsal fragment (QM F24685), cranial view (distal width 4.1 mm).

by almost 25 million years. They are consistent with a hypothesis of a southern origin for passerines. Little more can be discerned about the relationships of these fossils, nor can a palaeoenvironmental interpretation be made on the basis of their presence.

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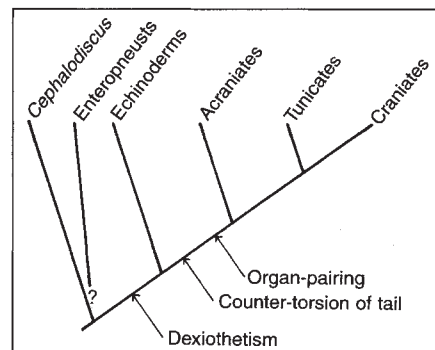
Dorsoventral axis inversion?

SIR — Arendt and Nübler-Jung in *Scientific Correspondence*¹ point out that the *decapentaplegic* gene is expressed ventrally in the fruitfly *Drosophila*, whereas the homologous gene *BMP-4* is expressed dorsally in vertebrates. They explain this paradox by supposing that an ancestor of vertebrates was bodily inverted in evolution, as long ago suggested².

Their suggestion is not compatible with the calcichordate theory of chordate origins³⁻⁶ (see figure). This theory implies that echinoderms and chordates are both descended from an ancestor like the extant pterobranch *Cephalodiscus* which lay right-side-downwards on the sea floor (dextiothetism), so that old right became new ventral. Subsequently, in the stem lineage of the chordates, the tail (stalk) rotated through 90° anticlockwise when seen from behind (counter-torsion of hind tail) which nullified dextiothetism in the tail. Counter-torsion did not affect the head. Instead there was later, in the head, a mirror-duplication on the right of organs (gill slits, left pharynx) which had previously existed only on the left. This organ-pairing still occurs in the metamorphosis of amphioxus.

Arendt and Nübler-Jung's paradox concerns organs derived from the calcichordate tail, where, because dextiothetism has been nullified by counter-torsion, chordate dorsal ought to be equivalent to insect dorsal. To explain it we suggest that chordate neurulation is a late phase of gastrulation. For in amphioxus and the tunicates the blastopore shifts forward during neurulation to become the neuro-pore at the anterior end of the animal while the internal cavity of the dorsal nerve cord long remains continuous with the archenteron by the neurenteric canal. We suggest that the expression pattern of *decapentaplegic* and its homologues may depend, not on inversion of the whole animal, but on the position of the gastrulation-neurulation process, being dorsal in chordates and ventral in insects.

Lacalli's suggestion⁷ that the chordate central nervous system originated by downward folding of dorsal ectoderm in an echinoderm-type larva is compatible



Phylogeny of deuterostomes according to the calcichordate theory with the successive origins of dextiothetism, counter-torsion of the tail and organ-pairing in the head. For further discussion see ref. 6. Amphioxus is an acraniate. The group Craniata here comprises Myxinoidea and Vertebrata (lampreys + gnathostomes).

with this explanation. In the calcichordate-head-derived parts of chordates, which were affected by dextiothetism but not by counter-torsion, the homologues of members of the *dorsal* cascade of *Drosophila* will probably control left-right rather than ventrodorsal pattern⁶.

Peterson⁸ argues, with good reason, that the ventral nervous system of enteropneusts is homologous to the central nervous system of insects. He also contends, however, that the enteropneust dorsal nervous system is homologous with the dorsal nervous system of chordates. This is doubtful, as the fossils indicate that the chordate central nervous system arose in the tail, with the brain situated where the tail joins the head. Adult enteropneusts have nothing corresponding to the tail. All parts of their nervous system are therefore too anterior to be homologous with the central nervous system of a chordate. Also, the enteropneust dorsal nervous system is not associated with a notochord or neurenteric canal. The dorsal nervous system of enteropneusts resembles the ectoneural nervous system of echinoderms in being intra-epidermal and centred in the mesosome, the left half of which is homologous with the echinoderm water vascular system.

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Searching for certainty

J. C. R. Hunt

The Weather Revolution: Innovations and Imminent Breakthroughs in Accurate Forecasting. By Jack Fishman and Robert Kalish. Plenum: 1994. Pp. 276. \$27.95.

The Scariest Place on Earth: Eye to Eye with Hurricanes. By David E. Fisher. Random House: 1994. Pp. 250. \$23.

WHEN even scientific journalists ask whether improvements in numerical weather prediction rely on empirical fixes, it is clearly high time that some good books should be written to explain to a wide audience the basis of recent progress in this great international endeavour. These two popular accounts go some way to meet this need. Both are written in journalistic prose, illustrated with a few useful line diagrams, and both provide informal histories of the scientific and technical discoveries that have led to modern meteorology and weather forecasting. Jack Fishman and Robert Kalish cover most aspects of weather forecasting and touch on aspects of global climate change, seasonal forecasting and the depletion (or 'hole') of ozone in the stratosphere. David E. Fisher, on the other hand, aims to cover a narrower theme in more depth, writing a story around his own experiences and those of other eye witnesses of Hurricane Andrew's impact on Miami in August 1992. The style of the books is reminiscent of O. G. Sutton's *Understanding Weather* (1960) or, more recently, James Gleick's *Chaos* (1989). But they contain hardly any discussion of the underlying mathematical ideas that are as relevant as the science and technology.

Fishman and Kalish nicely tell how modern weather forecasting has developed from empirical rules (whose 'unscientific' nature led the Royal Society to suppress forecasts in the United Kingdom from 1866 to 1877), to the application of physical principles (Buys Ballot's 'law' and the Bergen school's 'fronts' and 'air-masses') and then to the use of extensive computations, stemming from L. F. Richardson's visionary scheme of 1922, which became practical only with the arrival of von Neumann's ENIAC electronic computer at Princeton in 1945, running at about 10^2 floating point operations per second (FLOPS). Today's computer speeds are about 10^{10} FLOPS, with data from measurements ingested at about 10^8 bits per day. Both these rates are rising exponentially by roughly a factor of between 10 and 100 every 10 years.

Fishman and Kalish rightly emphasize the enormous increase in data that have become available for improving weather forecasts from new kinds of 'remote sensing' measurements and images obtained from weather radars and satellites. But

they might also have emphasized that, despite the enormous investment in these systems, their data are only now beginning to be effectively used for numerical predictions over longer periods, as a result of 'data assimilation' techniques originating from mathematical control theory. This is the first of the really new elements in the weather-forecasting revolution of the 1990s.

The second new element is the marked

Scientist, 28 January 1995, page 18).

Fisher describes the awesome power of hurricanes and the history of recent ones in the southeast Atlantic, some of which made landfall with devastating effect whereas others turned back or failed to develop. The physical principles of these vagaries are well covered.

Fishman and Kalish's last chapter, entitled "The revolution has begun", is an upbeat account of modern forecasting, especially at the 2-to-5-day period. They neglect to mention, however, that beyond this limited period it is impossible to make accurate deterministic forecasts, as E. Lorenz demonstrated in 1963, thus negating Richardson's 1922 prophecy of numerical prediction becoming as accurate as the nautical almanac. The authors unwisely remark that later mathematical discoveries will perhaps enable Lorenz's theoretical limitation to be overcome — I

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

Eye of the storm — satellite image of Hurricane Andrew.

improvement in modelling the small-scale but vitally important features of atmospheric motion that cannot be calculated explicitly in the Richardson-like models of the large-scale motions. In a sense, the small-scale models are based on Luke Howard's remarkable insight in 1802 (which long pre-dated modern discussion of 'order from chaos') that there are only a few important types (or, in the language of physics, 'eigenstates') of atmospheric motion on this scale, which are made visible in the form of cloud patterns (stratus, cumulus and so on) — a point mentioned but not emphasized by Fishman and Kalish.

Hurricanes are essentially another eigenstate of atmospheric motion: once triggered, they develop a characteristic structure that persists for many days. This means that a generic subgrid scale model, together with satellite-image data, may be used to calculate hurricane tracks. The main numerical weather-forecasting centres are making more rapid progress than suggested in these books (see *New*

wouldn't bank on it. In fact, the third new element in weather forecasting in the 1990s is the constructive recognition of this limit, so that forecasting is becoming more probabilistic, especially beyond about 5 days.

The weather interests everyone in different ways — as an exciting phenomenon to be described and classified, as a huge technical challenge involving measurements, forecasts and so on, or as a manifestation of many physical processes with complex mathematical behaviour, which overall are in a delicate state of dynamic, chemical and even biological balance. These authors have succeeded in popularizing the first two aspects. But I think they underplay and slightly misrepresent the theoretical concepts that are now guiding the development of modern meteorology, even in its most practical aspects. □

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Beyond the forest primaeval

Stuart L. Pimm

From Coastal Wilderness to Fruited Plain: A History of Environmental Change in Temperate North America from 1500 to the Present. By Gordon G. Whitney. Cambridge University Press: 1994. Pp. 451. £45, \$54.95.

"LEARN history to avoid repeating its mistakes" is advice that ecologists might value as much as politicians. Whitney's ecological history of the northeastern quarter of the United States is as much about our global future as it is about a local past. The history is that Europeans arrived, increased dramatically in numbers, moved inexorably westward and

impact on the land, first girdling the trees and then burning them. The colonists cleared the land in much the same way, but in much larger numbers. They learned empirically that different forests indicated very different soil fertilities, and they cleared the best land quickly and completely. Swine grazed the remaining forests and caused a massive decline in beech and oak. Free-ranging cattle retarded the forest's regeneration.

In England, high land value, cheap labour and high densities of farm animals to produce manure all encouraged crop rotation. Shortages of capital and labour quickly converted colonists to the new ways. Grains were often cultivated without manure, with yields only about 25 per cent of those in England. When yields fell, the farmers cleared new fields. Few businesses were as profitable as buying and selling land, moving westward and repeating the cycle.



The North American dustbowl: soil exposed by crop farming is quickly desiccated, then swept away by prairie storms. This picture is taken from *Habitats* by Tony Hare (Macmillan Publishing USA, \$25), a look at the world's 14 principal ecosystems — from rainforest to savanna, tundra to wetlands — through a series of fold-out panoramas that ingeniously blend photographic images of landscapes, flora and fauna. Each panorama is accompanied by several pages of text and explanatory diagrams. (Published as *Nature Worlds* in the United Kingdom by Macmillan at £14.99.)

replaced the Native Americans, their forests and their prairies. Perhaps by understanding this model we may change the present trends that otherwise will lead in the next century to the loss of our remaining grasslands and tropical forests.

What a place the pre-settlement forest must have been. The trees were often huge, and decaying woody debris covered the forest floor. Whitney's fine collection of old photographs is more compelling than any description. Who lived there? The conventional wisdom is only a few natives, treading lightly on the landscape. This is perhaps a western myth or, tragically if true, the consequence of western diseases. Epidemics in the early 1600s caused massive mortality in coastal populations. America was more a "a widowed land than a virgin land".

The surviving natives certainly had an

As late as 1850, much of the inland forest was still uncut. By 1860, lumber production was the second largest manufacturing industry. ('Cotton was king.') Lumbering cleared more land than agriculture after 1880. Initially, the cut selectively removed such valuable trees as white pine and red spruce. Later, the pulpwood industry clear-cut across large areas. Lumbering generated large amounts of waste, or 'slashings', that supplied the fuel for some of the continent's worst fires. These fires often destroyed the 'seed trees' left behind for regeneration and their fire-sensitive seedlings. By 1920, only around 4 per cent of the native forests remained. Maine and Pennsylvania retained less than 0.06 per cent of theirs.

The forests recovered, but they were often not the same. The selective removal

of valuable tree species contributed to their modern scarcity. Species with high reproductive rates, such as birch and aspen, have prospered. William Batram was bemoaning the disappearance of familiar species as long ago as 1764. In the familiar pattern, large mammals (particularly predators) were quickly hunted to local extinction. So too were beavers (for their fur) and vulnerable herbivores such as elk and bison. Species that depended on uncut forest, such as the ivory-billed woodpecker, became extinct.

Whitney's survey extends to the Mississippi, not the prairies beyond. East of the river, however, is a prairie peninsula encompassing much of Illinois and parts of neighbouring states. Prairies were hard-going for the settlers. There was little wood for houses and fences. Nonetheless, the destruction of the prairie was rapid (within 50 years) and complete (less than 1 per cent remains). In Illinois, less than 260 hectares of black-soil prairie remain of the original 6.6 million hectares. Wet prairies offered swarms of 'green-headed flies' and widespread malaria. Draining the peninsula's extensive wetlands solved these problems and land prices soared. Illinois, Indiana, Iowa, Missouri and Ohio drained more than 85 per cent of their wetlands and more than 99 per cent of Wisconsin's sedge meadows disappeared. Once-common prairie birds and butterflies now live only on the tiny remnants of virgin prairie.

Whitney's text lacks the easy, narrative style of William Cronon's 1983 *Changes in the Land* (which covers only New England) because the text is packed with the rich documentation of these changes. One remarkable botanical reconstruction is of the pre-settlement ranges of common trees, compiled from 150 references of land-survey records, some two centuries old. Certainly Whitney is a key to a large and often obscure literature; there are roughly 2,500 references. He also presents three themes having a powerful resonance with modern problems.

Setting priorities for managing biodiversity requires some sense of representation. We need more prairies, but even if we are relatively rich in forests, some forest types (and the plants and animals they house) are very much rarer now than in the past.

Some prairie communities may now be far too restricted to survive. We may try to restore them on abandoned farmland to extend their area, but what were those communities like? And what processes maintained them? The oak-savannas in Illinois may have been maintained by the natives burning them. Restoration may be impossible if we do not understand history.

Finally, a long list of external factors — the price of beaver skins and potash from burnt trees, the replacement of

open fireplaces with the efficient Franklin stove, the fires caused by the sparks from train fireboxes — changed the landscape briefly and dramatically. If change was rapid in the nineteenth century, how much more rapid will it be in the twenty-first? Perhaps Whitney's most useful lesson is that the factors altering the modern rates of tropical deforestation and the conversion of grasslands to agriculture will not only be complicated, but their relative importance is likely to fluctuate dramatically from decade to decade. □

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Mental tunnels

Martin Gardner

Inevitable Illusions: How Mistakes of Reason Rule Our Minds. By Massimo Piattelli-Palmarini. Wiley: 1995. Pp. 242. £19.99, \$27.95.

COGNITIVE psychologists study how we think and make decisions. In recent decades they have devised a vast array of confusing questions that most people answer incorrectly because of their poor grasp of logic and probability theory. The correct answers are so counterintuitive that they arouse strong emotions of disbelief comparable to those produced by familiar optical illusions.

Massimo Piattelli-Palmarini, a cognitive psychologist at the Massachusetts Institute of Technology, has written a delightful informal survey of what are known as 'cognitive illusions'. They arise, he says, because of curious blind spots, or mental tunnels, in our mind. Moreover, these tunnels often seriously distort our thinking in such areas as law, politics, economics and medical statistics.

The author's example of what he calls a "super tunnel" is a notorious brain teaser involving elementary probability. It generated such a storm of controversy when Marilyn vos Savant published it in her *Parade* magazine column that the *New York Times* (21 July 1991) reported the fuss on its front page. We can model the problem with three playing cards, one of which is an ace. The operator of the game shuffles the cards and places them face down. You put your finger on a card. The probability you have chosen the ace clearly is 1/3.

Suppose the operator, who knows where the ace is, removes a card that is not the ace. Two cards remain. Most people believe that the probability your finger is on the ace has now risen to 1/2. Wrong! It remains 1/3. Even more amazing is the fact

that if you shift your finger to the other card, the chances it is the ace doubles to 2/3. Savant was bombarded with thousands of letters, some from leading mathematicians, objecting to her correct answer.

Piattelli-Palmarini has a raft of other instances where intuitions lead one astray. Which is larger, the set of all seven-letter words ending with 'ing' or the set of all such words with 'i' in the fifth position? Obviously the second set is larger because it includes the first, yet on actual tests most people guess the other way.

Consider this statement:

Steve is very shy and withdrawn, invariably helpful, but with little interest in people or in the world of reality. A meek and tidy soul, he has a need for order and structure, and a passion for detail.

Which is more likely, that Steve is a librarian or a farmer? Most people pick librarian. Their blind spot is letting a librarian stereotype override the fact that there are far more farmers than librarians.

This neglect of background statistics is involved in other classics of cognitive research. A laboratory test is 79 per cent accurate in detecting a certain disease. The disease is known to affect only 1 per cent of the population. If you test positive, what is the probability you have the disease? The correct answer, which the author says can be established by Bayes' theorem (to which he devotes an informative chapter), is 8 per cent! Our intuition, we are told, is faulty because we have failed to consider the background information.

Are the cognitive psychologists right on this one? Let's raise the accuracy of the test to 100 per cent. Surely the background information is now totally irrelevant. Why would it become relevant if the test were, say, 99 per cent accurate?

The most tireless researchers on cognitive illusions are Amos Tversky, at Stanford University, and Daniel Kahneman, at Princeton. Is Piattelli-Palmarini right in saying their discoveries are so revolutionary that they deserve the Nobel prize in economics? Or should we agree with their leading detractor, the German psychologist Gerd Gigerenzer? He and others argue that the tricky problems posed by cognitive-illusion researchers represent rare, carefully contrived instances where intuitions are indeed unsound, but that their work on such illusions is making mountains out of molehills.

Whatever the case, *Inevitable Illusions* is the best popular book yet in this peculiar field. It will be of as much interest to recreational mathematicians as to psychologists and general readers. □

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Race to the swift

Lawrence Freedman

The Sorcerer's Challenge: Fears and Hopes of the Weapons of the Next Millennium. By David Shukman. Hodder and Stoughton: 1995. Pp. 256. £20.

IT has long intrigued me that in the cartoon battles between superheroes and supervillains that sully childrens' television, the villains always seem to have some device to track the heroes and watch their every move (something our military would love) yet when they actually shoot at a hero they invariably miss (which, by and large, our military do not once they have a fix on the target). It would not do, of course, for heroes to be struck down with ease, so much high-tech science fiction ends up by denying a basic feature of modern armed conflict in order to keep the story going.

Away from fantasy worlds, one cannot rely on the enemy being inept with a basic tool, especially an enemy that has demonstrated considerable technological sophistication elsewhere. Indeed, the best line available to a defence scientist worried about resource constraints is to warn of the consequences if the other side makes the crucial breakthrough first. This line served the defence research establishment well during the Cold War years, but has lost some of its appeal with the apparent loss of the former Soviet Union's demonic character, or at least its transformation into a power that has become incapable of mounting a serious non-nuclear threat to the West. Some of the most vivid descriptions in this book have David Shukman and his television crew teasing secrets out of destitute Soviet military laboratories, where grand ideas are kept alive by underpaid scientists, working in rooms with cracked light bulbs and broken locks, dreaming of access to US technology as part of some scheme of strategic cooperation.

The great merit of this account of the ideas and people in the world of defence research and development is that it provides a snapshot of the process of adjustment to the post-Cold War world. The snapshot comes after the euphoria immediately following the breach in the Berlin Wall and the heady days of Desert Shield and Desert Storm, and the frustrations of Somalia and Bosnia. The scientists have no shortage of ideas, but funding is tighter than ever and they must now justify them according to new political criteria, which for the moment seem to demand a virtually painless war.

If we are not fighting for the survival of our way of life, the casualties must be few and far between, methods must be found to provide defences against any

New in paperback

Powers of Ten: About the Relative Size of Things in the Universe by Philip Morrison and Phyllis Morrison. W H.

Freeman/Scientific American Library, \$19.95, £13.95. Based on a film made by the Office of Charles and Ray Eames for IBM, this book takes readers on a wonderful pictorial tour of the Universe using 42 consecutive scenes, each at a different 'power of ten' level of magnification.

The Strange, Familiar and Forgotten: An Anatomy of Consciousness by Israel Rosenfield. Picador, £5.99. The author

presents a variety of classical clinical cases — patients with amnesia, aphasia, multiple personalities, alien limbs — to support his ideas about how memory and consciousness work. Reviewed by Christopher Longuet-Higgins in *Nature* **360**, 117 (1992).

Speciation and the Recognition Concept: Theory and Application edited by David M. Lambert and Hamish G. Spencer.

Johns Hopkins University Press, £29. A collection of 17 papers on the new concept of species developed by Hugh E. H. Paterson in the 1970s. "The concept is claimed to remedy several problems with the earlier [biological species concept] and to aid research on

speciation. These claims do not survive close examination", argued Jerry A. Coyne in *Nature* **364**, 298 (1993).

The Limits of Safety: Organizations, Accidents, and Nuclear Weapons by Scott D. Sagan. Princeton University Press, \$14.95, £12.95. "Important and refreshing. . . ranges from the general theory of accidents to how-to-do-it suggestions for any nation's nuclear planners. It is a skilful blending of social, physical, organizational and military science and is highly recommended to readers in all four fields", wrote David L. Sills in *Nature* **367**, 30 (1994).

The Great Power-Line Cover-Up by Paul Brodeur. Back Bay Books (Little, Brown), \$12.95. The author, a writer at the *New Yorker*, alerted Americans in 1968 to the health hazards of asbestos. In this updated edition of a book first published in 1993, he now sets out to show "how the utilities and the government are trying to hide the cancer hazards posed by electromagnetic fields."

Order, Chaos, Order: The Transition from Classical to Quantum Physics by Philip Stehle. Oxford University Press, £22.50. "A serious exposition of an intellectually enthralling tale", wrote Brian Pippard in *Nature* **371**, 485 (1994).

measures an enemy might use to bring the war home to our own societies and, if possible, victory should be achieved with the minimum number of enemy casualties. Shukman therefore finds himself exploring ant-like robots deputizing for soldiers, methods of dealing with ballistic missile attacks, and nonlethal weapons such as gases that cause engines to break down. Meanwhile, much effort still follows the key developments of recent decades — towards a more accurate eye, impeded by neither night nor cloud, and methods of striking with precision so that the target is disabled with the minimum of effort and scant risk of collateral damage.

Journalists let loose among defence scientists are liable to produce awe-struck prose. Shukman is too experienced to be taken in by all the stories of the wizardry to come, although he might have been a little more sceptical about US ideas for 'brilliant pebbles' in space to intercept stray ballistic missiles. He is sufficiently unnerved to find himself caught on the horns of the classical dilemma of wondering whether resources and talents are being wasted while fearing the consequences if the breakthroughs are left to be exploited by more sinister forces. □

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Life in the slow lane

Peter D. Moore

The Private Life of Plants. By David Attenborough. BBC Books: 1995. Pp. 320, £17.95. To be published in the United States later this year by Princeton University Press.

PLANT-WATCHING, unlike bird-watching, has never really gripped the imagination of the general public. The reason, of course, is quite simple: plants don't behave. They simply don't do anything. At least, they don't do anything at the kind of pace that allows us to sit and watch them. Plant physiologists have long tried to convince us that plants are as sensitive to their environment as any other living organism and that they respond to the stimuli that surround them. But, although we have learned of such phenomena since our earliest biological training, few of us have been particularly impressed by all the tropisms, nutations, 'sleep movements' and touch sensitivities of plants that the textbooks describe. Animals, ourselves included, are such hasty creatures that we cannot actually appreciate the more leisured world of plants.

David Attenborough's BBC television series about the private life of plants shows it to be anything but pacific. The relentless struggle for existence involves

plants in a constant competitive battle that requires invasion, consolidation of position, establishing supply lines, sexual conquest, numerical enhancement, the enslavement of local animal populations and the development of foraging systems penetrating into neighbouring areas. All these behavioural techniques are displayed in the series by means of the most remarkable footage of time-lapse photography ever used to document these rarely watched aspects of the life of plants.

It is traditional, and necessary, that a book should be published to accompany the series, not only to provide a permanent record of some of the memorable pictures of plants, but also to act as a set of notes recording details of parts of the script and, most especially, the names and particulars of some of the examples shown on the screen. The popularity of such a publication is evidenced by the fact that this book has already reached the top of the UK bestsellers' lists. But can a traditional book format, however well illustrated, substitute for the mobile image that has succeeded in convincing us that plants really are living, even apparently sentient, organisms? Obviously it cannot, and those who expect the same level of spectacular imagery are bound to be disappointed. A still picture of a bent honeysuckle stem with the caption "plant searches for a hold on a neighbour" cannot encapsulate the fascinating film of spiralling, extending stems blindly groping for support.

But one should not expect this of a book. What one hopes to find is a text that is written in the same informative, lively, enthusiastic manner and with the same mixture of awe and excitement that characterizes the television script, accompanied by high-quality colour stills that can at least generate mobility in the reader's imagination. In this Attenborough has succeeded most admirably. As ever, he manages to combine readability and ease of style with academic respectability. Factual errors are scarce (pollen grains are certainly not 20–250 nanometers in diameter) and the temptation to anthropomorphize is generally avoided. Technical jargon is virtually absent, and I for one welcome this — botanists are too often guilty of hiding their ignorance behind a veil of pseudoscientific verbosity. Scientific names, however, add precision and the author has adopted the useful technique of including them in the index so that serious students can follow up his examples in more detail.

The book and the television series will open up the world of botany to a generation of budding biologists and lead many young scientists into this fascinating and fast-developing field. □

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The origin and early diversification of angiosperms

Peter R. Crane, Else Marie Friis & Kaj Raunsgaard Pedersen

The major diversification of flowering plants (angiosperms) in the Early Cretaceous, between about 130 and 90 million years ago, initiated fundamental changes in terrestrial ecosystems and set in motion processes that generated most of the extant plant diversity. New palaeobotanical discoveries, combined with recent phylogenetic analyses of morphological and molecular data, have clarified the initial phases of this radiation and changed our perspective on early angiosperm evolution, though important issues remain unresolved.

ANGIOSPERMS dominate the vegetation of most terrestrial ecosystems and consist of roughly 250,000–300,000 extant species, more than all other groups of land plants combined. For almost 150 years, understanding the origin and early diversification of these dominant land plants was hindered by what was thought to be an uninformative fossil record, uncertain relationships among extant angiosperms, and apparently insuperable morphological 'gaps' between angiosperms and other seed plants (gymnosperms). The 1960s and 1970s saw notable progress toward breaking this impasse. Syntheses of data from extant angiosperms demonstrated that the subclass Magnoliidae is a phylogenetically basal assemblage^{1–3} and studies of fossil pollen, leaves and reproductive structures documented a major diversification of angiosperms in the Early Cretaceous between about 130 and 90 million years before present^{4–9}.

Recently there has been renewed interest in the patterns and processes of early angiosperm evolution, and explicit phylogenetic analyses, based both on morphological and on molecular data, have clarified and revitalized many of the old debates^{10–14}. In the fossil record, bulk sieving techniques have yielded diverse and exquisitely preserved Cretaceous flowers (both mummified and charcoaled) that have resolved the systematic relationships of many early angiosperms and identified the source of important dispersed Cretaceous pollen^{15–19}. New information on floral form and reproductive biology in putatively basal extant groups has also catalysed comparative studies and enhanced the interpretation of fossil material^{20–22}. Here we review how these developments have sharpened arguments over the relationships among basal angiosperms and their seed plant relatives, and have changed our perspective on early angiosperm evolution.

Origin of angiosperms and their flowers

Ideas on the origin of angiosperms have sometimes invoked polyphyly, have treated the pteridosperms (seed ferns) as a natural group, and have implicated almost all groups of fossil and living gymnosperms as potential angiosperm ancestors^{9,23}. More recent work has emphasized cladistic discrimination of relationships among major clades of extant and extinct seed plants¹⁰ (Box 1). Parsimony analyses, based on morphological data, have shown that angiosperms are one of the most strongly supported monophyletic groups in the plant kingdom (see Box 1) and that the pteridosperms (as traditionally defined) are a highly unnatural group^{10,11}. However, the most striking result from recent phylogenetic analyses is the support for earlier ideas that identified Bennettitales (extinct) and Gnetales (extant; Fig. 5) as the seed plants that are most closely related to angiosperms (see Box 1). The resulting group (Bennettitales, Gnetales and angiosperms, plus *Pentoxylon* in some analyses) has been termed the 'anthophytes', to emphasize their shared possession of flower-like reproductive structures¹¹. Among extant taxa, angiosperm monophyly and a close relationship between Gnetales and

angiosperms (to the exclusion of *Ginkgo*, conifers and cycads), is also supported by parsimony analysis of partial 18S and 26S ribosomal RNA sequence data^{14,24} as well as analyses of combined morphological and molecular (*rbcL*, 18S, 26S) data^{14,25}.

Although all of the explicit cladistic studies conducted to date^{10,11,26–28} broadly support the anthophyte concept, they differ markedly in their resolution of relationships within the clade, and in their identification of the closest relatives of anthophytes (see Box 1). In addition, the long-standing question of whether angiosperm flowers are derived from a simple branch (euanthial) or derived from multiple branches (pseudanthial) is still unresolved (Fig. 1). The origin of angiosperm stamens and carpels, and their homologies with structures in other seed plants also remain problematic²⁹. Angiosperm stamens are both more regular and more simple than the pollen organs of the Bennettitales, Gnetales and most Mesozoic pteridosperms. In contrast, angiosperm carpels and bitegmic ovules are relatively complex compared to the ovulate structures of Gnetales and Bennettitales. Particular concerns are how the carpel and the outer layer of the typical angiosperm ovule should be compared to structures in related groups^{30,31}. Current explanations are all inadequate, and lean heavily on critical interpretations of fossil specimens that are not well understood (such as *Caytonia*, *corystosperms*).

Such uncertainties highlight the need to clarify structural and developmental homologies among the reproductive structures of angiosperms and related groups; in many respects, the morphological gap between angiosperms and other seed plants remains as wide as ever. This gap can be closed most directly through renewed morphological documentation and phylogenetic analysis of well preserved fossil gymnosperm material from Mesozoic fossil floras^{32,33}. Progress may also be possible by understanding how the genes controlling angiosperm stamen and carpel differentiation are expressed in the reproductive organs of extant gymnosperms (such as cycads, conifers, Gnetales)²⁹.

Angiosperm phylogeny and floral evolution

Current hypotheses of angiosperm evolution recognize two large clades (monocotyledons and eudicots) embedded within a poorly defined basal assemblage (grade) of magnoliid dicots (Magnoliidae¹). The monocotyledons are defined as monophyletic by their single cotyledon and other features³⁴. Eudicots are circumscribed by the production of triaperturate or triaperturate-derived pollen (convergent in Illiciaceae, Schisandraceae³⁵).

Generalizations developed around the turn of the century emphasized large multiparted flowers like those of extant *Magnolia* (Figs 1a and 2a) as a starting point for angiosperm floral evolution^{36,37}. Recent ideas accept that plants in the subclass Magnoliidae retain a broad array of probable unspecialized angiosperm features^{20,22} (such as parts generally free, lack of differentiation within the perianth), and have also documented

FIG. 1 Extremes of floral diversity in extant basal (magnoliid) angiosperms. *a*, Large multiparted flower of extant *Magnolia stellata* (Magnoliaceae), showing nine tepals and numerous stamens and carpels; magnification, $\times 1$. *b*, Small few-parted flowers of *Sarcandra glabra* (Chloranthaceae); each flower consisting of a single bract, carpel and stamen (photograph courtesy of P. K. Endress) $\times 3.8$. Interpretations based on recent phylogenetic results¹⁴ suggest that flowers of *Magnolia* and *Sarcandra* are, respectively, elaborated and reduced compared to the basic angiosperm condition. More radical interpretations of floral evolution suggest that all angiosperm flowers may not be homologous, and that the simple flowers of some (such as *Sarcandra*) are equivalent only to parts of flowers in others (such as *Magnolia*)^{44,86}.

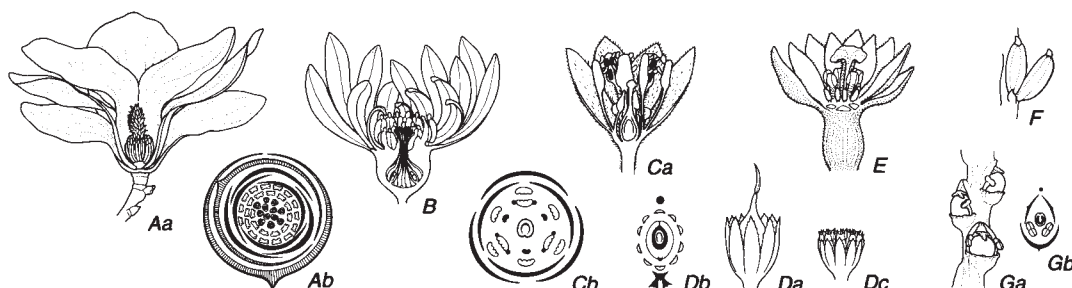
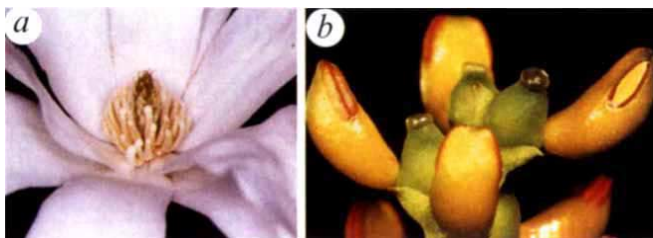


FIG. 2 Floral diversity in extant basal (magnoliid) angiosperms, illustrating variation in the number of carpels, stamens and perianth parts per flower. Generalized floral diagrams are shown in Ab, Cb, Db and Gb. A, *Magnolia* (Magnoliaceae); B, *Calycanthus* (Calycanthaceae); C, *Cinna-*

momum (Lauraceae); D, *Ceratophyllum* (Ceratophyllaceae) (*a*, pistillate; *c*, staminate flowers); E, *Hazomalania* (Hernandiaceae); F, *Ascarina* (Chloranthaceae), showing two staminate flowers; G, *Piper* (Piperaceae), showing three flowers.

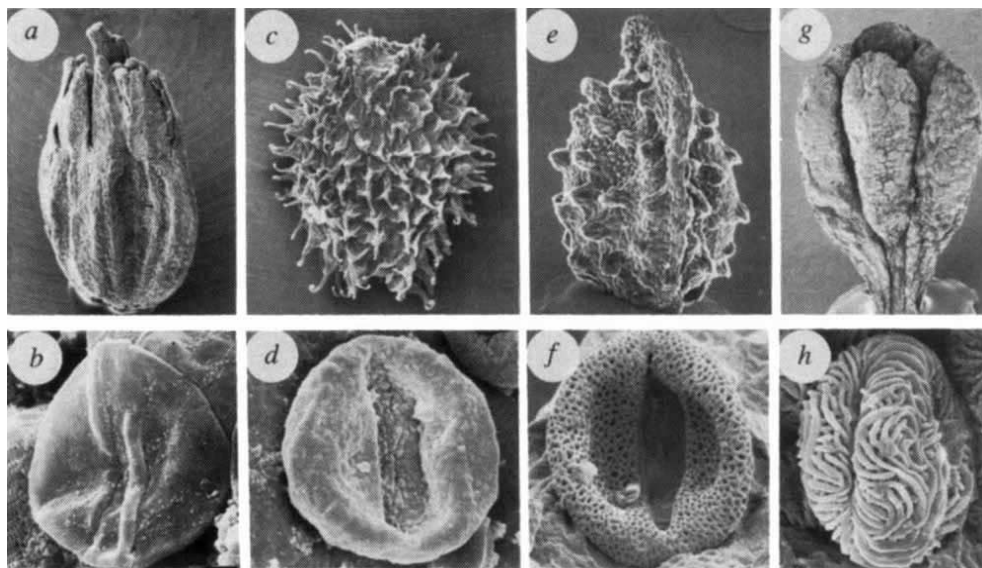
several previously unrecognized floral features that are general among extant magnoliids (such as valvate anther dehiscence³⁸, ascidiate carpel development³⁹). Modern studies have emphasized the great diversity of floral form, biology and structure among magnoliids^{22,40}. Variation in the number and arrangement of floral parts is extreme (Fig. 2), and both large, multiparted bisexual flowers and small, simple, frequently unisexual flowers are widespread^{22,40} (Fig. 1).

Identifying the likely basic condition of angiosperm flowers is therefore intimately connected with the recognition of phylogenetic patterns among magnoliids (Box 2). Unfortunately, the substantial morphological differences that separate angiosperms from all other seed plants, combined with uncertain relationships

among the anthophytes, makes it difficult to 'polarize' many of the crucial characters. This, in turn, complicates attempts to 'root' the angiosperm tree and contributes to the instability of current phylogenetic results^{12,14,41}. Among the studies currently available, there is emerging agreement that undue attention has focused on extant Magnoliaceae and its allies, and combined morphological and molecular results tend to favour phylogenetic models in which taxa with small, trimerous or even more simple flowers are basal in angiosperms^{14,42-45} (see Box 2).

Fossil evidence of early magnoliid diversity. The variety of leaves and pollen in the Early Cretaceous^{6,7} implies that magnoliids were diverse early in angiosperm evolution, and this is supported by the surprisingly rich, emerging record of fossil flowers

FIG. 3 Scanning electron micrographs of fossil early angiosperm flowers and carpels with their corresponding pollen. *a*, Epigynous flower showing tepals and protruding stamens, western Portugal, Early Cretaceous (Barremian or Aptian); magnification; $\times 20$. *b*, Monocolpate pollen with striate exine ornamentation from stamens of above type of flower (see *a*); $\times 2,200$. *c*, Unilocular fruiting unit covered with hooked unicellular hairs, and containing a single, orthotropous, pendulous seed; Potomac Group, Virginia, USA, Early Cretaceous (Early-Middle Albian); $\times 22$. *d*, Monocolpate *Transitoripollis*/*Tucanopollis*-like pollen from the stigmatic surface of above type of fruiting unit (see *c*); $\times 1,680$. *e*, *Couperites mauldinensis*, unilocular fruiting unit, showing protruding 'resin bodies', and containing a single pendulous, anatropous seed; Potomac Group, Virginia, USA, Late Cretaceous (Early Cenomanian); $\times 44$. *f*, Monocolpate *Clavatipollenites*-type pollen from the stigmatic surface of *Couperites mauldinensis* (see *e*); $\times 1,200$. *g*, *Spanomera mauldinensis*, staminate flower with five tepals and five



stamens; Potomac Group, Virginia, USA, Late Cretaceous (Early Cenomanian); $\times 47$. *h*, Tricolpate *Retitricolpites*-type pollen from stamens of *Spanomera mauldinensis* (see *g*); $\times 1,825$.

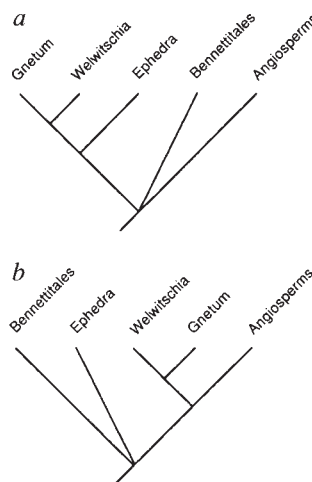
BOX 1 Hypotheses of relationship among angiosperms and related plants

Two contrasting hypotheses of phylogenetic relationships among anthophytes. Morphological characters supporting the anthophyte group include: the presence of an additional integumentary envelope, granular-columellate structure of the pollen wall and at least some stomata syndetocheilic^{10,11,27}. Other potential defining characters include scalariform pitting in the secondary xylem, whorled or opposite arrangement of microsporophylls, apical meristems with tunica-carpus organization, wood with syringal lignin subunits (Mäule reaction) and (secondarily) non-saccate pollen^{10,11,27}. New information on fertilization in *Ephedra* and *Gnetum*^{88,89} also raises the possibility that double fertilization may be a general feature of anthophytes. Monophyly of the Bennettitales is supported by the distinctive interseminal scales, and the aggregation of pollen sacs into bivalved synangia (present in all Bennettitales except a few Triassic taxa)^{10,72}. Monophyly of angiosperms is supported by sieve tubes and companion cells derived from the same initials, stamens with two pairs of pollen sacs, anthers with a hypodermal endothecium, microgametophyte of only three nuclei, carpel with stigmatic pollen germination, carpel enclosing a (probably) bitegmic ovule, megaspore wall lacking sporopollenin, megagametophyte with only eight nuclei and the formation of a triploid endosperm^{10,11}.

Uncertainty concerning relationships among anthophytes is introduced by: (1) possible secondary loss or transformation of critical morphological features (such as loss of syndetocheilic stomata in *Ephedra* and many angiosperms; loss of granular exine in most angiosperms); (2) the homologous or convergent origin of certain angiosperm-like features in Gnetales (especially in *Welwitschia* and *Gnetum*, Fig. 5) and Bennettitales (such as bisexual flowers); (3) the ambiguous homology of the second integument in the three anthophyte groups and the 'cupule' of potentially related taxa (such as *Caytonia*, corystosperms); and (4) uncertainty regarding the basic condition of many characters among basal angiosperms (such as orthotropous versus anatropous ovules, one-seeded versus many-seeded carpel, few-parted versus many-parted flowers).

a, Hypothesis A^{10,11,90}. Gnetales are monophyletic and the derived similarities of *Gnetum*/*Welwitschia* and angiosperms are interpreted as convergence (such as reticulate-veined leaves, reduction of male gametophyte, tetrasporic female gametophyte lacking archegonia and with free nuclei serving as eggs, cellular early embryogeny). This hypothesis is consistent with parsimony analyses of current molecular data (*rbcl*¹³, rRNA^{14,24}), as well as analyses of combined morphological and molecular data (morphology plus *rbcl*²⁵ and morphology plus rRNA¹⁴).

b, Hypothesis B²⁷. Gnetales are paraphyletic, with *Gnetum* and *Welwitschia* the sister group to angiosperms. This hypothesis interprets the derived similarities of *Welwitschia*, *Gnetum* and angiosperms as homologous.



(Fig. 3 and Box 3). In the extensive assemblages of Early Cretaceous angiosperm reproductive structures from Portugal and eastern North America, all of the fossils described so far are small (frequently less than 2 mm in length), and because they occur with larger fossils of other plants (such as conifers) it seems unlikely that this is a result of depositional bias. The flowers are generally few-parted and often with an undifferentiated perianth. Stamens generally have small pollen sacs with valvate dehiscence, and a relatively large apically expanded connective⁴⁶. Carpels generally have a poorly differentiated stig-

matic surface. Among magnoliids, the fossil record indicates that several extant families were already differentiated by about the Turonian (Box 3).

Fossil evidence of early monocot diversity. Among extant plants monocotyledons comprise only about 22% of angiosperm species. Over half of this diversity is accounted for by four families (Orchidaceae, orchids; Poaceae, grasses; Cyperaceae, sedges; Arecaceae, palms). The Cretaceous fossil record of monocotyledons is depauperate compared to that of contemporary magnoliids and eudicots, and perhaps reflects a variety of biases working

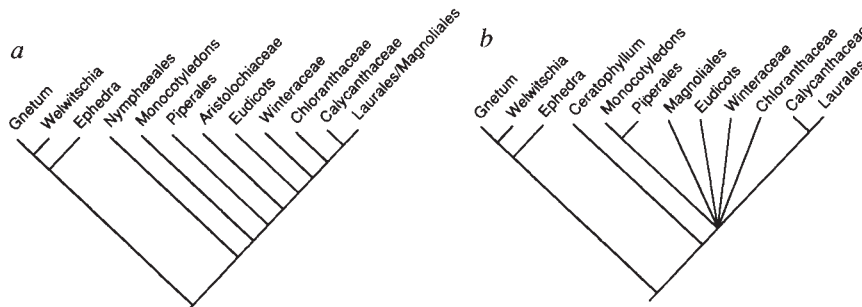
BOX 2 Hypotheses of relationships among basal angiosperms

Two of several published hypotheses of relationships among basal angiosperms. Taxa placed in a basal position by different recent studies include *Ceratophyllum*^{13,42}, Idiiospermaceae-Calycanthaceae⁴³, Chloranthaceae/Piperaceae^{44,45} and Nymphaeales¹⁴.

a, Hypothesis A¹⁴. Phylogenetic analysis of combined morphological and rRNA data showing relationships among extant Gnetales and basal angiosperms. Under this interpretation angiosperms are 'rooted' close to Nymphaeales, monocots are interpreted as an early branch in angiosperm evolution, and basal magnoliids are various more or less herbaceous taxa with basically simple flowers (secondarily complex in derived Nymphaeaceae). Magnoliales (including Annonaceae,

Degeneriaceae, Magnoliaceae, Myristicaceae) are placed in a relatively derived position. Eudicots are monophyletic and diverge from the basal magnoliid assemblage below Laurales/Magnoliales. Note that an alternative 'rooting' in the vicinity of Magnoliales would imply a relatively late divergence of monocotyledons from magnoliids.

b, Hypothesis B²⁵. Phylogenetic analysis of combined morphological and *rbcl* data showing relationships among extant Gnetales and basal angiosperms. Under this interpretation *Ceratophyllum* is the basal angiosperm lineage (not considered in hypothesis A) and eudicots are monophyletic, most other relationships are unresolved. Aristolochiaceae are not considered.



BOX 3 Fossil evidence of floral diversity among early angiosperms

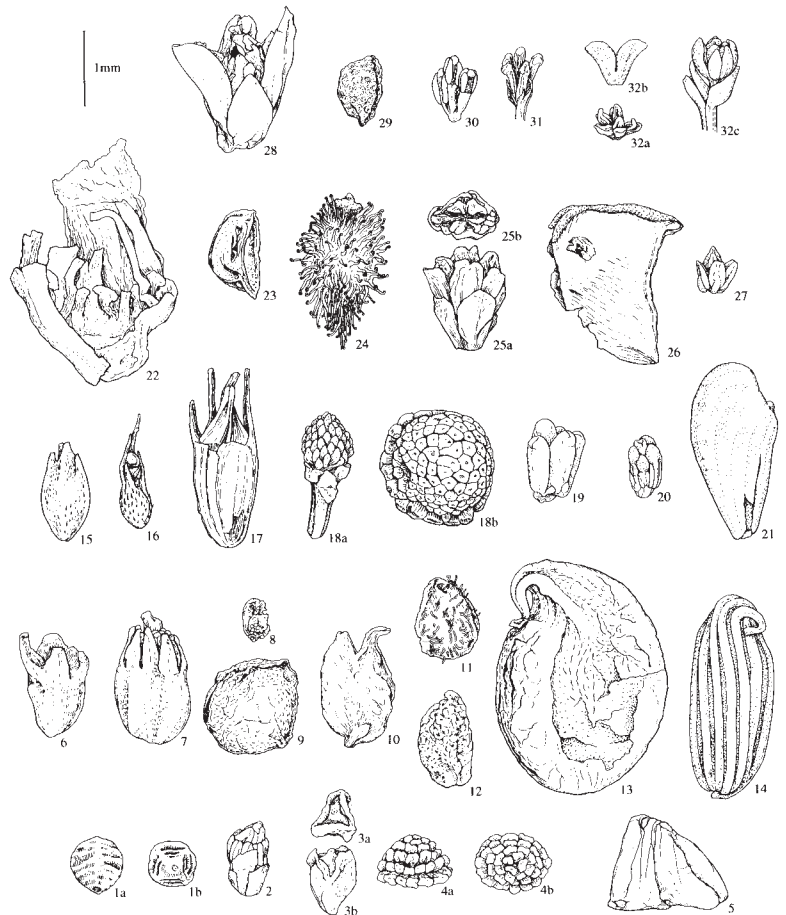
THE most extensive information on Early and mid-Cretaceous angiosperm reproductive structures is from western Portugal (1–14)¹⁹ and the Atlantic Coastal Plain of eastern North America (15–32)¹⁸ which together provide a sequence of floras ranging in age from Valanginian(?)–Hauterivian(?) to earliest Cenomanian.

Valanginian(?)–Aptian (1–14). Although only partially described, the angiosperm component of the earliest floras (Aptian and older, for example, 1–14) is dominated by small, simple flowers and flower parts. Epigynous flowers of possible lauralean affinity (such as 2, 3, 6, 7) and unilocular, single-seeded fruits or fruitlets with monocolpate pollen (such as 8, 9, 11–14) are especially common and diverse¹⁹. *Clavatipollenites*-type pollen, for which a chloranthoid affinity has been inferred⁹¹, occurs on the stigmatic surfaces of some of these fruits. *Clavatipollenites*-type pollen is also known *in situ* within distinctive angiosperm stamens and is widespread in dispersed pollen floras from the Early Cretaceous^{4,9}. Triaperturate pollen diagnostic of eudicots is also present¹⁹. Other putative angiosperm macrofossils from Aptian and older rocks include probable magnoliid leaves⁹² and horned (?*Ceratophyllum*-like) fruits of uncertain systematic affinity⁹³. One small specimen with attached leaves and reproductive structures, which is reported to show a mosaic of chloranthoid and piperales features⁹⁴, is too poorly preserved for detailed study of the flowers, fruits, stamens or pollen. In addition, dispersed pollen grains from Aptian and older rocks include forms perhaps related to extant Winteraceae³⁵ and Magnoliales⁹⁵.

Albian (15–27). In Albian floras (such as 15–27) small epigynous flowers (such as 15–17) and unilocular single-seeded fruits or fruitlets (such as 21, 23, 24) remain common. One of the most distinctive of these (24) appears to combine characters of Piperales, Chloranthaceae and perhaps Circaeasteraceae (Ranunculidae)¹⁸. A variety of other taxa are also present, including probable flowers of Lauraceae (22)¹⁸ and Calycanthaceae⁹⁶, possible magnolioid fruits (such as 18) and stamens¹⁸, and possible fruits of *Ceratophyllaceae*⁹³. Flowers of basal eudicots become a distinctive component of Cretaceous floras from the mid-Albian onwards and several lineages of 'lower' hamamelidids can be recognized during the mid-Cretaceous phase of angiosperm diversification, including Platanaceae^{16,52} (such as 25, 31) and perhaps trocho-dendroids^{5,7,51,52} and buxoids^{52,97} (such as 32) (see Fig. 3g, h).

Earliest Cenomanian (28–32). By the early part of the Late Cretaceous the magnoliid groups include chloranthoids⁹⁸, and Lauraceae (such as 28)⁹⁹, both of which were apparently geographically widespread. Probable Magnoliaceae¹⁰⁰ were also present. Eudicots of this age include a variety of platanoids^{16,53}, rosiids¹⁷ and other taxa²⁰.

Fossils illustrated are from the Valanginian(?)–Hauterivian(?) of Torres Vedras, western Portugal (1–5); the Barremian or Aptian of Catefica, western Portugal (6, 12), Famalicão, western Portugal (13, 14), and Vale de Agua, western Portugal (7–11); the Early or mid-Albian of Puddledock, Virginia (15–22, 24), Bank near Brooke, Virginia (25), and Kenilworth, Maryland, (23); the Late Albian of West Brothers, Maryland (26, 27, 30, 31); and the Early Cenomanian of Mauldin Mountain, Maryland (28, 29, 32). 1, Four-angled fruit or seed in lateral (a) and apical (b) view. 2, Epigynous (?) flower with monocolpate, reticulate pollen. 3, Epigynous *Hedyosmum*-like flower in apical (a) and lateral (b) view. 4, Floral structure with several whorls of stamens



(?unistaminate flowers) in lateral (a) and apical (b) view. 5, Group of seeds. 6, Four-angled, epigynous structure^{18,19}. 7, Epigynous flower with monocolpate, finely striate *Cabomba*-like pollen (see Fig. 3a, b)¹⁹. 8, 9, 11, Unilocular single-seeded fruiting units with strengthening tissue in seed wall; 8 and 11 have adhering *Clavatipollenites*-type pollen. 10, Bicarpetate fruit. 12–14, Unilocular single-seeded fruiting units with strengthening tissue in fruit wall. 15–17, Epigynous floral structures. 18, Multiparted floral structure; smaller specimen in lateral view (a), larger specimen in apical view (b). 19, Apocarpous pistillate? flower. 20, Staminate flower. 21, Unilocular fruit with strengthening layer in fruit wall. 22, Fragment of lauralean flower. 23, Unilocular single-seeded fruiting unit with strengthening tissue in the seed wall and with monocolpate pollen. 24, Spiny, unilocular and single-seeded fruiting unit (see Fig. 3c, d). 25, Pistillate flowers of *Platanocarpus brookensis* in lateral (a) and apical (b) view⁵³. 26, 27, Fruitlet (26) and staminate flower (27) of *Spanomera marylandensis*⁹⁸. 28, Trimerous flower of *Mauldinia mirabilis*⁹⁹. 29, Fruit of *Couperites mauldinensis* with adhering *Clavatipollenites*-type pollen (Fig. 3e, f)⁹¹. 30, Chloranthoid androecium⁹⁸. 31, Pistillate flower of *Platanocarpus marylandensis*¹⁶. 32, Staminate (a) and pistillate (b, c) flowers of *Spanomera mauldinensis* (Fig. 3g, h)⁹⁷.

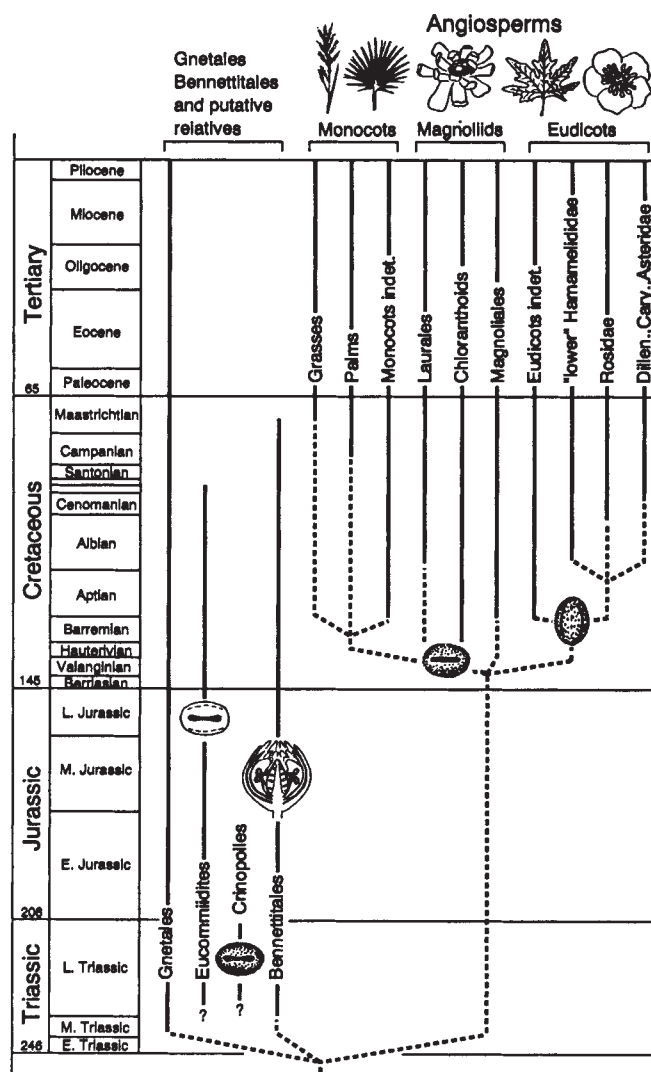
against both their preservation and recognition. During the mid-Cretaceous, monocots are probably represented by several leaves⁴⁷, and more equivocally by dispersed pollen^{48,49}. However, evidence of rapid monocot diversification is provided later in the Cretaceous by fruits of Zingiberales (gingers and their allies), and leaves and stems of palms³⁴. By the Early Tertiary many monocot groups had differentiated^{34,50} (Fig. 4).

Fossil evidence of early eudicot diversity. Eudicots comprise about 75% of extant angiosperm species, and are recognized as a monophyletic group in phylogenetic analyses of combined morphological and molecular data^{14,25}. Triaperturate pollen, which is diagnostic of the group, is first recorded in dispersed

palynological assemblages around the Barremian–Aptian boundary (about 125 Myr) or perhaps slightly earlier.

Basal groups in the eudicot clade include the ranunculids (Ranunculidae, sometimes included in the Magnoliidae) and also the 'lower' hamamelidids^{51,52}. Embedded within this basal eudicot assemblage are the diverse groups that comprise the bulk of extant angiosperms, including the 'higher' hamamelidids along with the subclasses Rosidae, Caryophyllidae, Dilleniidae and Asteridae, which contain many highly diverse extant families (such as Asteraceae, the sunflower family). Among the eudicots that can be recognized during the mid-Cretaceous diversification are several 'lower' hamamelidid lineages and a diversity of

FIG. 4 Simplified phylogenetic tree showing the relative stratigraphic ranges of selected angiosperm groups and putative close gymnosperm relatives. The fossil record of Gnetales is based primarily on diagnostic dispersed ribbed pollen, with supporting evidence from rare Triassic^{72,87} and Cretaceous⁷² macrofossils. The fossil record of *Eucommiidites* is based primarily on dispersed pollen, with seeds and pollen organs known from the Jurassic and Cretaceous³². The combined evidence suggests that *Eucommiidites*-producing plants are closely related to the anthophyte group³². Crinopolles pollen is reported from the Late Triassic⁵⁷ but other organs of the plant are not known. Bennettitales have an extensive fossil record based on leaves and reproductive structures^{10,72}. The bennettitalean flower-like reproductive structure illustrated is *Williamsoniella*. Monocolpate pollen is the basic condition in angiosperms that is retained by monocots and magnoliid dicots. Triaperturate pollen is diagnostic of eudicots. Dillen, Dilleniidae; Cary., Caryophyllidae.



generalized rosiid types (Fig. 4). By around the Campanian, eudicots were very diverse^{20,50,52}.

Pre-Cretaceous angiosperms?

Current studies of the fossil record show an orderly sequence of appearance of angiosperm remains beginning with putative magnoliid pollen in the Valanginian, and triaperturate pollen of eudicots around the Barremian–Aptian boundary (or slightly earlier). By the early Cenomanian there are diverse magnoliids, a variety of hamamelidids and a few rosiids, which show clearly that several angiosperm families had already differentiated. By the Campanian, Maastrichtian and Palaeocene, many extant angiosperm families in all subclasses had already differentiated^{34,50,54}.

Claims of pre-Cretaceous angiosperms need to confront this orderly sequence, and although the literature on angiosperm origins is replete with putative pre-Cretaceous angiosperms, most have been shown to be stratigraphically misplaced, unrelated to angiosperms, lacking in diagnostic characters, or too poorly preserved for reliable determination⁵⁵. Recently, however, there has been renewed discussion of the possibility of pre-Cretaceous angiosperms stimulated by the anthophyte hypothesis (which implies that the lineage leading to angiosperms diverged from other known groups of seed plants before the Late Triassic)^{10,11,41}, the description of new and potentially relevant fossils from Jurassic and Triassic rocks^{56,57}; and calculations of divergence times based on molecular clock assumptions^{58,60}. These developments have highlighted the

importance of distinguishing clearly between the timing of angiosperm divergence (splitting of the stem lineage from its sister groups) and the timing of angiosperm diversification (splitting of the crown group into extant clades)⁴¹.

In our view, the absence of distinctive triaperturate pollen grains in numerous, rich, Triassic and Jurassic palynofloras from both hemispheres precludes the long cryptic period of evolution implied by some estimates of rates of molecular evolution, at least for eudicots⁶¹. The systematic affinities of recently described putative Jurassic and Triassic angiosperms^{56,57,62,63} are either clearly with other groups (such as dipteridaceous ferns⁶⁴) or remain equivocal (such as Triassic *Sanmiguelia*-like plants and Crinopolles-type pollen grains^{64,65}). Although some of these fossils may be attributable to the anthophyte clade, they highlight the difficulties of accurate systematic determination based on inadequate material. Most defining features of angiosperms (Box 1) are unlikely to be preserved in the fossil record. The most useful diagnostic morphological features of angiosperms are stamens with two pairs of pollen sacs, and a carpel enclosing the ovule. Both can only be observed in well preserved fossil material.

Coevolutionary interactions

Angiosperm diversification has often been linked to the diversification of pollen and nectar-collecting insects on the supposition that insect pollination provides new possibilities for reproductive isolation and therefore elevation of speciation rates^{66,68}. Compared to wind pollination, which is widespread in other seed

FIG. 5 Gnetales consist of only three extant genera: *Ephedra* (a, about 40 species in arid and semiarid areas); *Welwitschia mirabilis* (b, one species restricted to the Namibian Desert); and *Gnetum* (c, about 30 species of tropical rain-forest lianes and small trees). Despite the obvious morphological differences the group is widely regarded as monophyletic (Box 1) and is united by multiple axillary buds, opposite/decussate phyllotaxis (also reflected in the reproductive structures), circular bordered pits in the protoxylem, vessels (?derived independently to those in angiosperms) and a feeder in the embryo^{10,11}. In addition, *Welwitschia* and *Gnetum* share several derived characters that are thought to be convergent with similar features in angiosperms^{10,11} (see Box 1).



plants, insect pollination may also permit effective outcrossing at lower population densities and in a greater range of environments, perhaps thereby reducing extinction rates^{66–68}. These factors may explain the diversity of some of the most speciose angiosperm families (such as orchids), but the extent to which they provide a general explanation for the massive mid-Cretaceous increase in angiosperm diversity is uncertain^{69,70}. In the Bennettitales, flower-like reproductive structures provide indirect evidence of insect pollination from the Late Triassic to Late Cretaceous^{71,72}, and insect pollination may also have been established in some Mesozoic pteridosperms³³. The size and morphology of diverse mid-Cretaceous gnetalean pollen, combined with evidence from extant taxa⁷³, is also suggestive of insect pollination in the Gnetales. Early angiosperms may therefore have coopted pollinators from previously established relationships with other groups of seed plants.

Whatever the effect of insect pollination on the initial angiosperm diversification, discoveries of Early and mid-Cretaceous flowers leave no doubt that early members of the group were insect pollinated. Stamens in fossil flowers have small anthers with low pollen production, anther dehiscence is valvate, pollen grains are often covered with a pollenkit-like material, stigmatic surfaces are generally unelaborated and pollen grains are often smaller than the most effective size for wind dispersal^{18,19}. Comparison with modern relatives suggests that these flowers were probably pollinated by pollen-collecting or pollen-eating insects. Flowers pollinated by nectar-collecting Hymenoptera and Lepidoptera occur in more derived groups of angiosperms and appear later in the fossil record¹⁷.

Although Early Cretaceous angiosperms may have been very similar to their living relatives in pollination syndrome, modes of fruit and seed dispersal were probably substantially different²². Cretaceous angiosperm fruits and seeds are generally very small compared to their modern relatives, and there is no evidence of specialized mammal or bird dispersal. Among basal groups, as in angiosperms as a whole, the evolution of fleshy fruits, arillate seeds and other apparent adaptations for animal dispersal, seems to be correlated with the evolution of frugivorous/granivorous birds and mammals, perhaps during the latest Cretaceous, but most strikingly during the Early Tertiary. The only suggestion of animal dispersal of angiosperms in the Early Cretaceous is provided by a single species of small fruits covered with hooked spines (Box 3, Fig. 3c).

Rise to ecological dominance

The mid-Cretaceous diversification of angiosperms marks the transition from Mesozoic ecosystems dominated by ferns, conifers, cycads and Bennettitales to more modern Late Cretaceous and Tertiary ecosystems dominated by angiosperms^{74,75}. In the palaeobotanical record this change is much more profound than that occurring at the Cretaceous–Tertiary boundary and is also reflected in the transition from sauropod-dominated to ornitho-pod-dominated dinosaur faunas⁷⁶. Data on the floristic composi-

tion of both macrofloras and microfloras show that angiosperms had attained diversity levels of about 50–80% by the end of the Cretaceous⁷⁴, but there is evidence that angiosperm abundance still remained subordinate to gymnosperms and ferns in some habitats, perhaps over large geographical areas^{77,78}. The extent to which this is a general phenomenon is currently uncertain but it is supported in part by the palynological data and by the prominence of certain open-habitat ferns in some Late Cretaceous floras. These considerations emphasize the value of complementary macrofossil-based, and pollen/spore-based, assessments of diversity and abundance in evaluating the rate and magnitude of the angiosperm radiation. They also raise the possibility that disturbance by herbivorous dinosaurs may have been a significant ecological factor in the first half of angiosperm history⁷⁶.

Problems of large-scale stratigraphic resolution make it difficult to resolve geographic patterns in the angiosperm radiation but compilations of palynological data, especially from the Northern Hemisphere, show that the initial increase in angiosperm diversity occurred in low palaeolatitude areas^{75,77}. This result is of biogeographic interest for understanding the current distribution of relictual magnoliid families, and also has significant ecological implications. During the Early Cretaceous, low latitude areas experienced semiarid or seasonally arid conditions that may have promoted a weedy life history with precocious reproduction (progenesis)^{1,11,41}. Associated effects may have included simplification and aggregation of sporophylls to form a flower, enclosure of ovules in a carpel, truncation of the gametophyte phase of the life cycle, and major reorganization of leaf and stem anatomy. In turn, several of these features may have contributed to angiosperm ‘success’ through elevated speciation rates^{11,41,79} and/or more rapid and more flexible vegetative growth^{80,81}. It remains uncertain, however, whether such effects were manifested at the base of the angiosperm clade or within one or more angiosperm subgroups^{82,83}.

Interestingly, the Gnetales, which show many remarkable structural and biological convergences to angiosperms (Fig. 5), also diversified during the mid-Cretaceous in low palaeolatitude areas, although they never became significant at middle and high palaeolatitudes and experienced rapid decline during the earliest Late Cretaceous^{75,77}. The temporal, palaeogeographic, and perhaps ecological, parallels between this radiation of Gnetales and angiosperms implies a common response to changing environmental conditions. Explanations of angiosperm diversification may therefore have underemphasized the effects of environmental changes during the Early and mid-Cretaceous, which included high rates of sea-floor spreading, high sea-level stands and probably high global temperatures⁸⁴.

Future directions

The five living groups of seed plants are a poor sample of the total historical diversity of the seed plant clade and thus additional molecular phylogenetic analyses are likely to lead only to

limited progress in evaluating the phylogenetic relationships of angiosperms. However, reducing the gap between angiosperms and their gymnosperm relatives is important because of its implications for rooting the angiosperm tree. Renewed palaeobotanical efforts with the early members of the Gnetales, Bennettitales, angiosperms and other potentially closely related groups are therefore a high priority. Recognition of 'stem group' taxa, with some but not all of the defining features of the 'crown group' has been possible in other studies of land plant phylogeny, and would help to resolve several outstanding problems.

Current data from the fossil record and combined morphological/molecular phylogenies of extant taxa challenge the view that earliest members of the angiosperm clade were large, woody plants with *Magnolia*-like flowers. Instead they suggest a very different concept of early angiosperms as perhaps herbaceous plants of small stature. Flowers would have been small, simple (perhaps unisexual) and probably lacking clear differentiation into sepals and petals (suggesting that molecular genetic models of floral morphogenesis developed for the eudicots *Arabidopsis* and *Antirrhinum*⁸⁵ may require modification among magnoliids).

Stamens would have had a poorly developed filament and a well developed anther with valvate dehiscence. Pollen would have been small, monolete, tectate with a weakly developed endexine in non-aperturate areas. The gynoecium would have been composed of one or more unilocular carpels containing one or two ovules. The stigmatic surface would have been unelaborated.

Ecologically, the early diversity of angiosperms at low palaeolatitudes, and the parallel Aptian–Cenomanian radiation of angiosperm and gnetalean pollen in these areas, suggest that both groups responded in similar ways to the same environmental cues. Possible linkages between mid-Cretaceous climatic, tectonic and other environmental changes, and their possible impact on terrestrial ecosystems need to be explored. Early Cretaceous fossil plants from low palaeolatitudes are likely to be particularly informative. □

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Seismic and geochemical evidence for large-scale mantle upwelling beneath the eastern Atlantic and western and central Europe

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Seismic tomography and the isotope geochemistry of Cenozoic volcanic rocks suggest the existence of a large, sheet-like region of upwelling in the upper mantle which extends from the eastern Atlantic Ocean to central Europe and the western Mediterranean. A belt of extension and rifting in the latter two areas appears to lie above the intersection of the centre of the upwelling region with the base of the lithosphere. Lead, strontium and neodymium isotope data for all three regions converge on a restricted composition, inferred to be that of the upwelling mantle.

THE size and shape of mantle upwelling regions provide important constraints on the thermal budget of the Earth and on models of mantle dynamics. Most hotspot tracks are no more than 200–400 km wide, suggesting that upwelling regions are often relatively narrow features, of the order of a few hundred kilometres or less. But a number of geophysical observations, such as geoid and topographic anomalies and seismic tomography studies¹ indicate that at least the upper portion of upwelling regions can be significantly larger. Here we present evidence from seismic tomography, isotope geochemistry and regional geology which suggests that volcanism over a large region of the Earth's surface is related to a single, large-scale upwelling of the mantle. This region of upwelling resembles an inclined sheet and extends to depths greater than 500 km beneath the eastern Atlantic. It has a width of approximately 2,500 km in the NNE direction and bends to the east with decreasing depth for about 4,000 km beneath northern Africa, the western Mediterranean and central Europe (Fig. 1).

Regional geology

In this study, we suggest that the Eastern Atlantic volcanic province, the Western Mediterranean volcanic province and the Central European volcanic province (Fig. 1) may ultimately be derived from a common sublithospheric mantle source. The Eastern Atlantic volcanic province forms an elongate belt of islands and seamounts, which parallels the coast of northwest Africa and the Iberian peninsula. This province extends for approximately 2,500 km in the NNE direction from the Saharan seamounts and the Canary Islands in the south, to the Madeira Islands in the centre to the Biscay abyssal plain in the north. Subaerial volcanism in the Canary and Madeira islands began in the Early Miocene epoch^{2,3}, but submarine volcanism may have begun before 60 Myr (refs 4, 5). The western Canary and Madeira islands are situated on oceanic lithosphere, whereas the lithosphere beneath the eastern Canary Islands is transitional between oceanic and continental⁶.

The Central European volcanic province extends from France to southwest Poland to Hungary and includes the Massif Central in France (where volcanism occurred between 0 and 20 Myr and

between 35 and 65 Myr), the Rhenish massif (Eifel) and Rhine graben in Germany (0–20 Myr), Lower Silesia in Poland (15–40 Myr) and the western Pannonian basin in Czechoslovakia and Hungary (Eocene–Miocene and 1–12 Myr). Most of the volcanism in central Europe is related to a NNE-trending rift belt formed in the Early Cenozoic era⁷. This continental rift belt includes the Rhone, Limagne, Bresse, Rhine, Ruhr and Leine grabens. In contrast to other central European volcanism, Pannonian basin volcanism is associated with back-arc spreading⁸.

The Western Mediterranean volcanic province includes the Roman province of Italy (Quaternary period), the Aeolian Islands (Quaternary), eastern Sicily (Late Cretaceous–Quaternary), the Straits of Sicily (late Miocene–Holocene) and Sardinia and Corsica (Oligocene–Pleistocene)⁸. Each of these volcanic regions is underlain by continental crust which has been undergoing extension, as evidenced by intensive block-faulting and rifting. During the late Oligocene–early Miocene, the Balearic, Valencia and Algerian rifts formed in the western Mediterranean, extending the NNE-trending rift belt in central Europe to the southwest for a total length⁸ of more than 2,000 km. Subduction of the African plate occurred throughout the Tertiary period beneath much of the western Mediterranean, and continues to the present day beneath the Aeolian Islands.

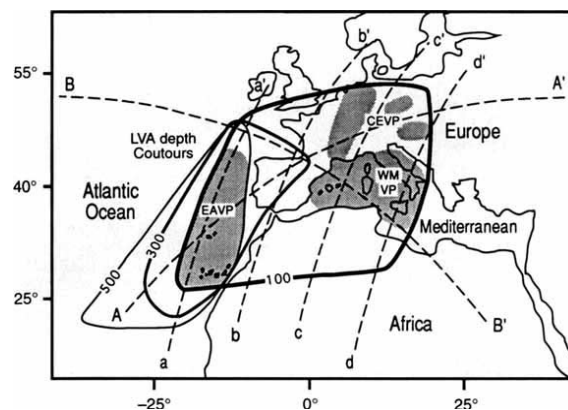
Seismic tomography

Figures 1 and 2 show results from the global upper-mantle S-wave velocity model RG5.5^{9,10}. A low S-wave velocity anomaly (LVA), with a width of about 2,500 km in the NNE direction and elongated about 4,000 km in the ESE direction, underlies the eastern Atlantic, northern Africa, the western Mediterranean and central Europe. The LVA in this model is a planar feature with a NNE strike and a westward dip.

Similar features are also found in other seismic models. Figure 3 shows NNE S-wave velocity cross-sections of the global model SH12/WM13¹¹ with the same locations as the profiles in Fig. 2b. Although their resolutions differ, both models are consistent with the presence of a large-scale, low-velocity anomaly extending from the eastern Atlantic to northern Africa, the western Mediterranean and central Europe. Local S-wave velocity models^{12–14} show low-velocity regions at depths shallower than 200 km beneath western and central Europe and the Mediter-

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FIG. 1 Map view showing the extent of the low (less than the global mean) S-wave velocity anomaly (LVA) at depths of 100, 300 and 500 km. Where the LVA intersects the base of the lithosphere, it covers an area of $2,500 \times 4,000$ km, extending from the Eastern Atlantic volcanic province (EAVP) to northern Africa, the Western Mediterranean volcanic province (WMVP) and the Central European volcanic province (CEVP). The LVA becomes narrower at depth, as is indicated by the 300- and 500-km depth contours, and dips to the WNW. Volcanic provinces are denoted by shading. The locations of seismic profiles in Figs 2 and 3 are shown with appropriately labelled dashed lines.



reanean (results of local models not shown due to limited coverage of study area). Local P-wave velocity models EUR85A and EUR89A^{15,16} indicate low-velocity regions at depths of 150–250 km beneath western and central Europe and the Mediterranean, whereas P-wave velocity model EUR89B¹⁶ shows low-velocity regions between 95 and 195 km.

The correspondence in location and pattern of major anomalies for S- and P-wave tomographic models is striking, and provides evidence for a large-scale low-velocity region in the upper mantle underlying the eastern Atlantic, northern Africa,

the western Mediterranean and western and central Europe. This conclusion is emphasized when we consider that many factors, such as quality and quantity of data sets, nonlinearity inversion approach, depth- and lateral-resolution differences, and dependence of velocity on temperature and volatile content, can affect inversion results for different models¹⁶. We interpret this low-velocity region to be a relatively hot (possibly volatile-enriched) region of mantle upwelling.

An important question concerns the detailed structure of upwelling beneath the study area. Is the LVA a single feature,

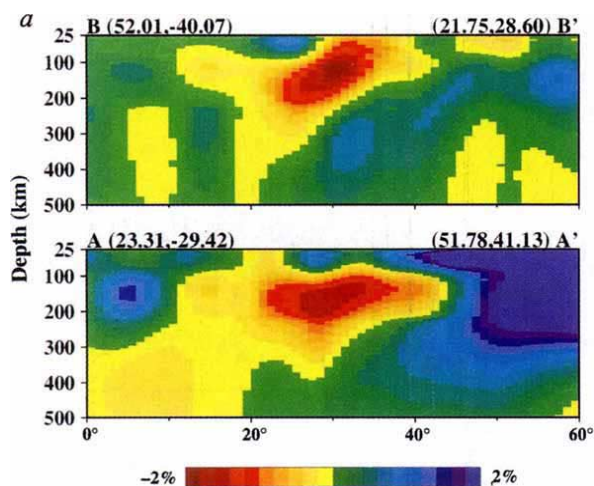
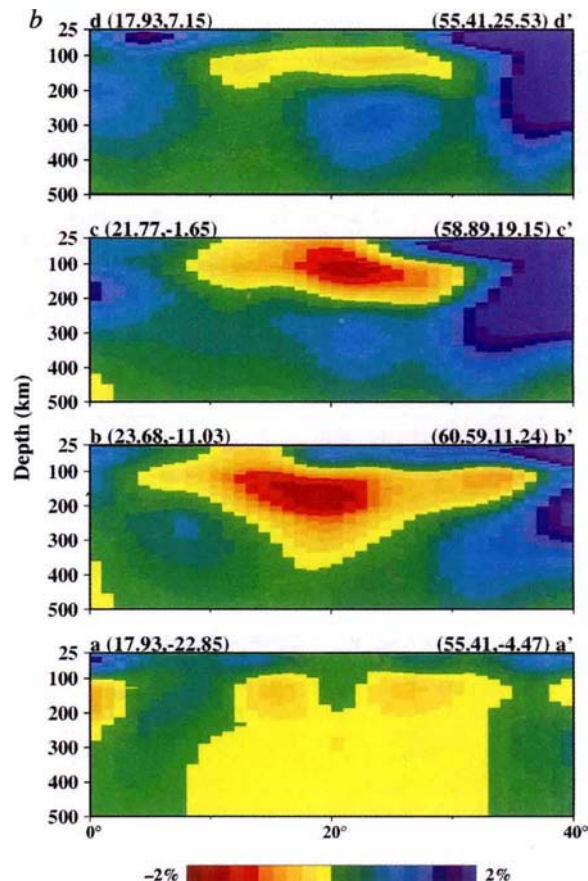


FIG. 2 Depth slices from 25 to 500 km for S-wave seismic velocity model RG5.5^{9,10}: a, in the northeast and southeast directions; b, in the NNE directions (profile locations shown in Fig. 1). The sheet-like LVA strikes to the NNE and dips to the WNW. It is $\sim 2,500$ km along strike, has a maximum thickness of 500–800 km, and extends to depths >500 km beneath the Eastern Atlantic volcanic province (EAVP). The centre of the sheet encounters the base of the lithosphere some 2,000 km to the east of the EAVP beneath the central European–western Mediterranean rift system. The two smaller low-velocity maxima between 100 and 200 km depth beneath the eastern Atlantic (profiles BB' and aa') may result from secondary instabilities rising from the inclined sheet at 200–300 km depth. A similar concept has been proposed for inclined plume conduits, but on a smaller scale^{37,48}. The fast velocity anomalies in profiles BB' (30° – 50°) and dd' (18° – 30°) may reflect stacking of subducted African oceanic lithosphere, which is being subducted with a northwest dip beneath the Aeolian Islands. Model RG5.5, obtained from surface-wave data, has a horizontal resolution of $\sim 1,000$ km and a vertical resolution of ~ 50 km at 50 km depth, and 300 km at 400 km depth; thus, this model cannot resolve subducted slabs thinner than 100 km. Velocity anomalies (see colour scale at bottom of panels a and b) are given in percentage of the global mean



at each depth, contoured at 0.4% intervals. Endmember coordinates, at the top of each profile, are given as (latitude, longitude) in degrees of a great circle. The horizontal axis is the distance in degrees ($1^{\circ} \equiv 111$ km) from the first latitude and longitude specified at the top of each section.

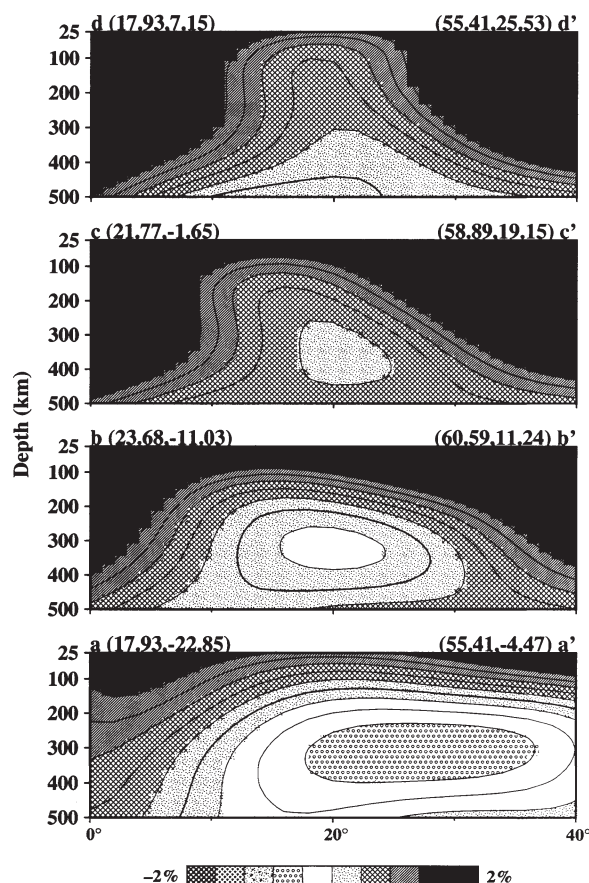
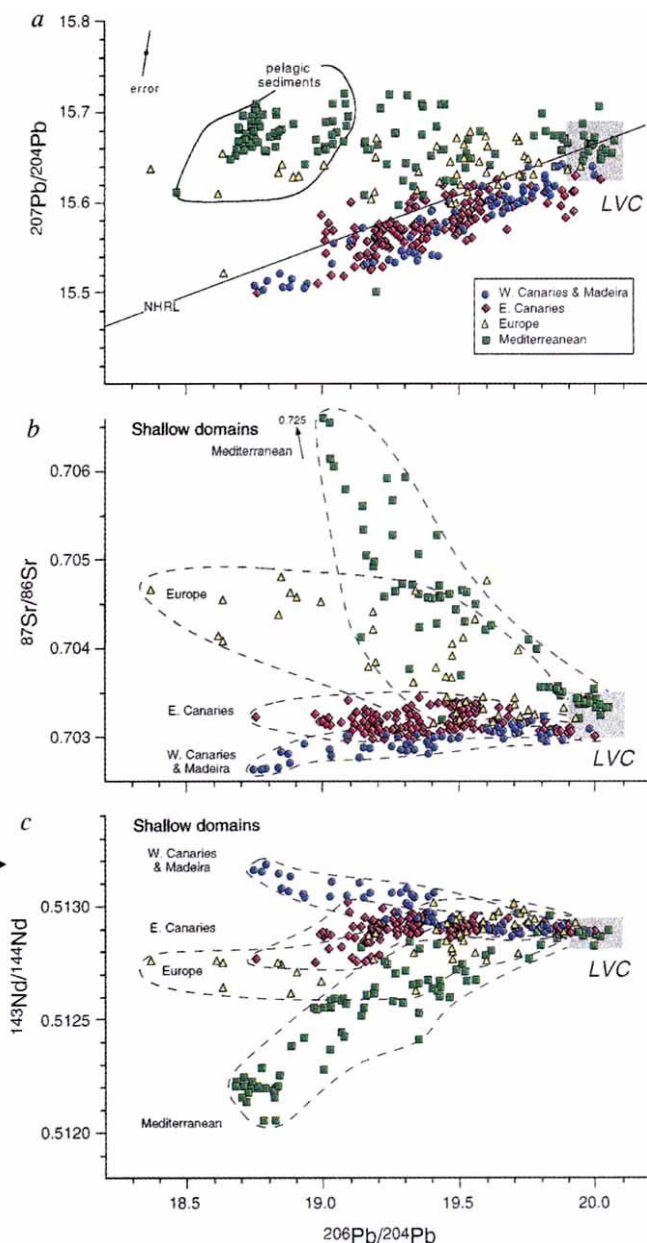


FIG. 3 North-south depth slices through the S-wave velocity anomaly in model SH12/WM13¹¹, which have the same locations as the depth slices in Fig. 2b. Model SH12/WM13 has increased resolution at greater depths (lower-mantle) than model RG5.5, but only one-third the resolution at shallower depths (upper-mantle). Nevertheless, the low-velocity anomaly in model RG5.5 is clearly seen in model SH12/WM13. Model SH12/WM13 shows the largest low-velocity anomaly at depths of 250–400 km beneath the eastern Atlantic. In contrast, model RG5.5 with its improved resolution at shallower depths shows the largest low-velocity anomaly beneath Europe and the Mediterranean. See Fig. 2 legend for additional information.

FIG. 4 Plots of $^{206}\text{Pb}/^{204}\text{Pb}$ against $^{207}\text{Pb}/^{204}\text{Pb}$ (a), $^{87}\text{Sr}/^{86}\text{Sr}$ (b) and $^{143}\text{Nd}/^{144}\text{Nd}$ (c) for Cenozoic volcanic rocks from four geographical regions: (1) eastern Atlantic oceanic domain, which includes the western Canary and Madeira islands (circles); (2) eastern Atlantic continental margin domain, consisting of the eastern Canary Islands (diamonds); (3) central European continental domain, including mafic volcanic rocks and carbonatites from the Massif Central, Rhenish massif, Rhine graben, Eger graben and Pannonian basin (triangles); and (4) western Mediterranean domain, containing Italy, Sicily, the Aeolian Islands and islands in the Straits of Sicily (squares). The isotope data from these four geographical domains form fan-shaped patterns. The data array for each region converges on a common composition with $^{206}\text{Pb}/^{204}\text{Pb} \approx 19.9\text{--}20.1$, $^{207}\text{Pb}/^{204}\text{Pb} \approx 15.62\text{--}15.68$, $^{87}\text{Sr}/^{86}\text{Sr} \approx 0.7030\text{--}0.7034$ and $^{143}\text{Nd}/^{144}\text{Nd} \approx 0.51282\text{--}0.51294$ ($\epsilon_{\text{Nd}} = 3.5\text{--}5.9$). This component is interpreted as having the composition of the low-velocity anomaly shown in Figs 1–3 and is therefore referred to as the low-velocity component (LVC). For each geographical region, the component(s) with lower $^{206}\text{Pb}/^{204}\text{Pb}$ is/are located in the lithosphere or shallowest asthenosphere. Possible endmember compositions for the shallower components are indicated in b and c. These shallow regional domains may primarily reflect (1) depleted oceanic lithosphere and asthenosphere (western Canary and Madeira islands), (2) transitional oceanic/continental type lithosphere (eastern Canary Islands), (3) lower crust and/or lithospheric mantle affected by ancient subduction (central Europe), and (4) upper crust and/or lithospheric mantle and asthe-

or does it reflect an averaging of spatially isolated low-velocity regions, such as a cluster of plumes which are smaller than the resolution of model RG5.5? Local P-wave model EUR89B¹⁶ shows laterally separated anomalies beneath Europe and the western Mediterranean, although we note that models EUR85A¹⁵ and EUR89A¹⁶ (with only slightly different reference models) for the most part do not. Whether the laterally separated anomalies in EUR89B represent multiple small plumes, however, cannot be resolved with this model, because the depth resolution over most of the area of interest (150–400 km) is relatively poor¹⁶. The recent local S-wave model EUR-S91^{13,14} has excellent reported lateral and depth resolution. This model shows the LVA to be continuous within the stated resolution,



osphere influenced by recent subduction (western Mediterranean). The Northern Hemisphere reference line (NHRL)⁴⁹, the field for deep-sea (pelagic) sediments^{50,51} and standard error due to mass fractionation of 0.5‰ per atomic mass unit are shown in a. Standard errors for $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ are generally smaller than the height of the symbols. Only data from samples analysed for Sr, Nd and Pb isotopes are shown. Data sources are refs 6, 18–26, 28, 31 and 52–58.

and does not show any evidence for deep roots of the LVA beneath central Europe or the western Mediterranean, in good agreement with global model RG5.5 used here.

Nevertheless, some degree of lateral discontinuity in the LVA is to be expected. It could result from localized up- and downwellings near the lithosphere–asthenosphere interface, or rising blobs and pipes of hot and/or volatile-rich mantle surrounded by cooler asthenospheric mantle entrained at shallower depths¹⁷. Although detected by volcanological and petrological methods¹⁷, such mantle structures, with diameters of less than 100 km, are beyond the resolution presently available with seismic tomography. The isotope geochemistry of Cenozoic volcanic rocks from regions overlying the LVA provides additional evidence that the LVA is a large-scale region of mantle upwelling.

Isotope geochemistry

Isotope data from Cenozoic volcanic rocks overlying the LVA are shown in Fig. 4. The data have been subdivided into four groups based on regional domain: (1) eastern Atlantic oceanic domain (western Canary and Madeira islands), erupted on oceanic lithosphere; (2) eastern Atlantic continental margin domain (eastern Canary Islands), erupted on lithosphere which is transitional in character between oceanic and continental; (3) central European domain, erupted on continental lithosphere and apparently affected to some degree by ancient subduction associated with the Hercynian orogeny¹⁸; and (4) western Mediterranean domain, a region influenced by recent subduction. The data for each group form trends crudely consistent with binary mixing. The arrays for each region converge on a restricted compositional range, which we interpret as being the isotope composition of the seismic low-velocity anomaly and henceforth refer to as the low-velocity composition (LVC).

The $^{206}\text{Pb}/^{204}\text{Pb}$ ratio for volcanic rocks from the eastern Atlantic oceanic domain correlates positively with $^{207}\text{Pb}/^{204}\text{Pb}$, $^{208}\text{Pb}/^{204}\text{Pb}$ (not shown) and $^{87}\text{Sr}/^{86}\text{Sr}$, and negatively with $^{143}\text{Nd}/^{144}\text{Nd}$ (Fig. 4). The $^{87}\text{Sr}/^{86}\text{Sr}$ ratio correlates negatively with $^{143}\text{Nd}/^{144}\text{Nd}$ (not shown). Lavas from the western Canary Islands have isotope compositions that overlap the LVC^{6,19}, whereas some lavas from Madeira have isotope compositions similar to average Atlantic normal mid-ocean-ridge basalts (N-MORBs) (ref. 20 and K.H., unpublished data). A lithospheric mantle source has been proposed for this depleted Madeira endmember^{20,21}, although a shallow asthenospheric origin cannot be excluded. Mixing of the LVC and shallow depleted mantle can account for the range of isotope compositions in the western Canary and Madeira islands.

The eastern Canary Islands have isotope compositions which extend from the LVC to higher Sr and lower Nd and Pb isotope ratios (Fig. 4). The mafic ($\text{Mg}\# > 62$ where $\text{Mg}\# \equiv 100 \text{ Mg}/(\text{Mg} + \text{Fe})$), low- SiO_2 ($< 44 \text{ wt}\%$) lavas from the eastern Canary Islands have isotope compositions similar to those of the western Canary Islands and the LVC. Lavas with higher SiO_2 ($> 44 \text{ wt}\%$) and/or lower $\text{Mg}\#$ (< 62) have less radiogenic Pb and more radiogenic Sr than the LVC. Correlations between the degree of differentiation and isotope ratios indicate a shallow origin for the component with unradiogenic Pb, presumably located within the lithosphere^{19,6}.

Mafic volcanic rocks from central Europe show continuous, negative trends for Sr versus Nd and Pb versus Sr, and a positive trend for Pb versus Nd (Fig. 4b, c). As $^{206}\text{Pb}/^{204}\text{Pb}$ decreases, these data approach the compositions of deep-sea sediments on the Pb–Pb isotope diagram (Fig. 4a). The arrays for these mafic volcanic rocks are generally consistent with mixing between two components¹⁸. Component A, which has major- and trace-element characteristics of melilitite nephelinite and isotope characteristics of the LVC, is interpreted as having a sublithospheric origin^{18,22}. Component B has $^{87}\text{Sr}/^{86}\text{Sr} \approx 0.705$, $^{143}\text{Nd}/^{144}\text{Nd} = 0.5125$, and lies above the Northern Hemisphere reference line (NHRL) at $^{206}\text{Pb}/^{204}\text{Pb} \approx 18.0$. It has trace element and isotope compositions similar to granulite and hydrous spinel peridotite

xenoliths entrained within the magmas, and probably has a lithospheric origin^{18,23}.

Volcanic rocks from the western Mediterranean domain define curvilinear Pb–Sr and Pb–Nd isotope arrays (Fig. 4). These volcanic rocks can be divided into two groups based on isotope composition and tectonic setting. Sodic ($\text{Na}_2\text{O}/\text{K}_2\text{O} > 0.7$) lavas from Mt Etna, Sicily^{24–26}, Straits of Sicily²⁷ and Pietre Nere, southeastern Italy²⁸ have Sr–Nd- and Pb-isotope ratios that overlap the LVC. Potassic and calc-alkaline lavas from regions influenced by recent subduction of the African plate (western Italy and the Aeolian Islands) extend to very high $^{87}\text{Sr}/^{86}\text{Sr}$ and to low $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{206}\text{Pb}/^{204}\text{Pb}$ ratios. Lavas from western Italy have the most extreme isotope compositions, with values similar to deep-sea sediments (Fig. 4a), whereas lavas from the Aeolian Islands fall in the middle of the array. Oxygen-isotope data and positive europium anomalies in samples from western Italy provide additional evidence that the contamination involves a sedimentary component, probably subducted to shallow depths in the upper mantle^{29,30} and located within the upper crust²⁹. Minor amounts of a third, MORB-like component are also seen in some lavas from the Aeolian Islands³¹, and in ancient tholeiites from Mt Etna^{24,26}.

The isotope data for the regions situated above the LVA (the eastern Atlantic, central Europe and the western Mediterranean) all converge to a narrow compositional range. This low-velocity composition (LVC; see earlier) has $^{87}\text{Sr}/^{86}\text{Sr} \approx 0.7030$ – 0.7034 , $^{143}\text{Nd}/^{144}\text{Nd} \approx 0.51282$ – 0.51294 ($\epsilon_{\text{Nd}} = 3.5$ – 5.9), $^{206}\text{Pb}/^{204}\text{Pb} \approx 19.9$ – 20.1 , $^{207}\text{Pb}/^{204}\text{Pb} \approx 15.62$ – 15.68 , $^{208}\text{Pb}/^{204}\text{Pb} \approx 39.60$ – 39.90 , and is interpreted to represent a sublithospheric mantle source beneath each geographical region. The LVC is readily distinguished from the mantle source for N-MORB, in that it has relatively radiogenic $^{206}\text{Pb}/^{204}\text{Pb}$ and $^{87}\text{Sr}/^{86}\text{Sr}$, and lower $^{143}\text{Nd}/^{144}\text{Nd}$ compared to most MORB. Hotspot volcanism in the regions surrounding the LVA, such as the Ahaggar, Cameroon Line, Azores, Iceland and the Cape Verde Islands, have compositions which are similar to some of the volcanic rocks above the LVA, but none of the isotope trends for these hotspots converge precisely on an endmember with the composition of the LVC.

As noted from the tomography, an important question concerns the structure of the LVA. Does it represent one large upwelling region or multiple smaller, discrete upwellings? Evidence for the presence of the LVC is found in all tectonic environments above the LVA, including: (1) regions associated with rifting and extension, such as the NNE rift belt running through central Europe (including the Massif Central, the Rhenish massif and the Rhine graben), the Eger graben, and the straits of Sicily; (2) regions associated with subduction, such as western Italy and the Aeolian Islands; (3) back-arc basins, such as the Pannonian basin; (4) hotspots, such as the Canary Islands, Madeira Islands and possibly Mt Etna. The ubiquitous presence of the LVC over such a large region irrespective of tectonic regime is a potent argument that the LVA represents a common mantle source reservoir with a distinguishable isotope composition. The fact that this isotope composition is relatively uniform, and distinct from the mantle source for MORB, also suggests a deep origin from a well mixed reservoir. The combined evidence from seismic tomography and isotope geochemistry is therefore consistent with the interpretation that the LVA is a common source reservoir for the least-contaminated volcanic rocks found in the region extending from the eastern Atlantic to central Europe and the western Mediterranean.

Regional tectonics and distribution of volcanism

A long-standing problem in the regional tectonics of Europe has been how to explain the opening of the western Mediterranean basin through extension, rifting and sea-floor spreading, even though the African/European plate boundary has been undergoing compression during the Eocene and early Miocene³². This problem is well illustrated by the southward rifting of the Sardinia–Corsica ridge and the Balearic ridge away from France and

Spain, respectively, at the same time that Africa was colliding from the south. We find that the slowest seismic velocities, and presumably the hottest mantle material, are located beneath the Gulf of Valencia and the North Balearic basin, which separate the Sardinia-Corsica ridge and the Balearic ridge from the mainland. This correlation suggests a causal relationship between the extension and underlying thermal anomaly.

Volcanism is typically absent in continental regions undergoing compression and crustal and lithospheric thickening, such as in the Atlas Mountains, the Alps, the Pyrenees and along most of the Iberian peninsula, but is common in areas of crustal extension. The most voluminous volcanism in Europe is associated with the central European rift system, which extends NNE from the western Mediterranean to northwest Germany. Although poorly understood, the origin of lithospheric thinning and rifting in central Europe must have been influenced in part by the late stages of the Alpine orogeny^{18,33}. Uplift and alkaline magmatism (in addition to more limited tholeiitic magmatism) associated with this extension, however, are difficult to explain solely through lithospheric thinning and seem to require a thermal anomaly in the underlying asthenospheric mantle^{18,34}. Indeed, elevated heat flow from the mantle beneath the central European rift belt has been proposed as the cause of subcrustal upwarping and rifting³³. The seismic and geochemical observations presented here are consistent with this interpretation. The NNE extensional belt, extending some 2,000 km from northwest Germany to the Algerian rift⁸, parallels the trend of the upwelling sheet observed in the seismic tomography, and also for the most part overlies the slowest part of the velocity anomaly in model RG5.5. This correlation raises the intriguing possibility that the extensional belt may be the surface manifestation of the intersection of the upwelling sheet with the base of the lithosphere, with the surface location being modified to some degree by the effects of the Alpine, Pyrenean and Atlas orogenies.

The Eastern Atlantic volcanic province has the most voluminous volcanism. There has been a long-standing debate as to whether or not the origin of the Canary Islands is related to tectonic structures, such as the South Atlas fault, or to a mantle hotspot³. Several observations suggest that the Canaries are related to an asthenospheric feature rather than a lithospheric one. These include the lack of geophysical evidence for large fractures beneath the Canary Islands³; the east-west age progression, in the direction of plate motion, observed in both the subaerial (>20 to 0 Myr) and submarine (>60 to 3–4 Myr) volcanic sequences¹⁷; and evidence³⁵ that the parental magmas on Gran Canaria are derived from depths greater than 100 km. The NNE trend of the Eastern Atlantic volcanic province, which parallels the trend of the extensional belt of central Europe and the western Mediterranean, and the location of this volcanic province above the deep roots of the upwelling sheet, provide strong evidence for a connection with the underlying asthenospheric mantle.

Origin of the LVA

In contrast to three-dimensional models of mantle convection which show downwelling in sheets and upwelling as columns³⁶, the LVA appears to be sheet-like in model RG5.5, striking to the NNE and dipping to the west. As discussed above, planar upwelling is consistent with the large-scale tectonic features, in particular the NNE-trending rift belt extending through central Europe and the western Mediterranean, and with the distribution of volcanism in the area above the LVA. The westward dip of the LVA may reflect a combination of eastward asthenospheric flow¹⁷ and lithospheric drag³⁷. The areal extent of this upwelling sheet along the base of the lithosphere (2,500 by 4,000 km) is about twice that estimated for plume heads in the mantle^{34,38}. White and McKenzie³⁴ proposed that plumes consist of a narrow (150–200 km across) central column, and a wide (1,000–2,000 km diameter) mushroom-shaped head of anomalously hot mantle (100–200 °C above ambient). Large heads (800–1,200 km

diameter) have been predicted^{38,39} for starting plumes derived from the core-mantle boundary, which may flatten to about twice their original diameter as they approach the overlying lithospheric plates. These models, however, are based on thermal plumes fed by narrow cylindrical conduits and so are not directly applicable to the feature discussed here.

Volcanism in each of the volcanic provinces can be traced back to the early Tertiary/late Cretaceous^{4,5,8,18}, suggesting that the mantle upwelling feature may be relatively long-lived. Considering the possible age and large size of this upwelling region, an important question is why continental rifting in central Europe has not progressed further, and why volcanism has not been more widespread. One possible explanation is that the temperature anomaly of this feature is not as large as that associated with hotspots such as Hawaii, or those which have produced flood basalts.

Plume heads that produce flood basalts are believed to be derived from the core-mantle boundary. High ³He/⁴He ratios at hotspots such as Hawaii and Iceland are consistent with derivation of at least some upwelling material from the deeper mantle^{40–42}. Volcanic rocks from Mt Etna and the Canary Islands, however, have relatively low ³He/⁴He ratios, with a range of 5.8–7.5 *R*_A (refs 26, 42, and D.G., K.H., J. Lupton and H-U. Schmincke, manuscript in preparation). Furthermore, volcanic fluids from Italy and Europe show no evidence for ³He/⁴He ratios above MORB values^{44–46}. Therefore, either the low-velocity anomaly is exclusively an upper-mantle feature, or it has been disconnected from a deep source for a sufficiently long time that the ³He/⁴He has been lowered by radiogenic ingrowth.

Available evidence from seismic tomography for a lower-mantle origin is equivocal. Global models SH12/WM13 and RG5.5 show this upper-mantle low-velocity anomaly extending only to depths of approximately 600–700 km (near the base of the transition zone in the upper mantle). Several seismic models^{11,47}, however, show a large-scale, low-velocity feature in the lower mantle farther to the south. If the LVA is just an upper-mantle feature fed from the boundary layer between the upper and lower mantle, this could explain why more extensive volcanism in Europe and the Mediterranean is absent and why continental rifting is still in its incipient stage.

These observations bear on the relationships between surface geochemistry, mantle dynamics and plate tectonics. Models for mantle convection have generally assumed that hotspots are underlain by thermal anomalies with a cylindrical geometry, and that the scale of these upwelling regions is considerably smaller (200–400 km in diameter) than discussed here. Our study provides evidence for the existence of large, sheet-like upwellings which should be considered in models of volcanism, mantle convection and regional tectonics. □

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LETTERS TO NATURE

Avalanche mixing of granular solids

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THE production of many goods, ranging from pharmaceuticals and foods to polymers and semiconductors, depends on reliable, uniform mixing of solids. Although there have been several notable recent advances^{1–6}, solid mixing processes are still poorly understood. We can neither qualitatively nor quantitatively determine the effectiveness of any given mixing process in advance. In contrast to the case of liquid mixing⁷, we do not have a widely accepted theoretical basis that describes the mixing of solids. Moreover, we cannot determine whether a given set of solids will mix or separate during a specified stirring process^{8–23}. As a step towards uncovering the basic physical principles, it is helpful to analyse systems that are both experimentally and theoretically tractable. Here we describe a geometric technique for the analysis of slow granular mixing processes, as are commonly encountered in industry. By comparing our calculations with experiments on thin rotating containers partially filled with coloured particles, we demonstrate that the mixing behaviour of powders in slow flows can be divided into geometric and dynamic parts. For monodisperse, weakly cohesive particles, geometric aspects dominate.

As an example of a system to be modelled, consider an upright two-dimensional disk partially filled with coloured passive particles and rotating about its axis (Fig. 1). For slow rotation, the surface layer mixes through the action of successive avalanches. Slow mixing implies that each avalanche stops completely before a new one begins. The avalanche duration scales as $\sqrt{D/g}$, where D is the container diameter and g is the acceleration due to gravity, so we require $\Omega\sqrt{D/g} \ll 1$, where Ω is the rotation rate. Material below the surface layer rotates as a solid body with the disk.

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Experimentally, the disk is thin enough for the dynamics to occur in a plane. We note that although individual grains in the experiment may take a three-dimensional path during an avalanche, we observe through the front and back of the glass disk that the macroscopic features—boundaries, streaks and so on—extend entirely through the material layer, and consequently large-scale structures remain planar and the experimental flow may be assumed to be two-dimensional. The particles used are dyed table-salt grains—cubes with a mean side length of 0.6 mm—and the disk is 240 grains in diameter and 40 grains deep. An avalanche occurs when the surface slope exceeds $\theta_i \approx 60^\circ$, after which the surface returns to its angle of repose, $\theta_r \approx 52^\circ$, as sketched in Fig. 1; for salt we measure $\theta_i - \theta_r = 8^\circ \pm 2^\circ$.

The detailed mechanism for avalanches is not entirely understood and has been the topic of considerable research^{1–6,24,25}. However, these details are not important here. The crucial point is that the surface motion that causes mixing is iterative; that is, one avalanche looks much like another, and the process of mixing can be represented as successive, nearly identical, avalanches which are repeated over and over.

The idea of the model is as follows. Consider any closed and slowly rotating mixing vessel, partially filled with granular material. Industrial examples of this kind of mixer include the V-blender³, the tube or drum mixer, and the rotating kiln. The crucial observation is that irrespective of the detailed dynamics of the avalanche itself, the result of the avalanche is to transport an initial wedge of material (dark grey in Fig. 1) downhill to a new wedge (white in Fig. 1). Thus we can divide the problem of avalanche mixing into two distinct parts: transport of wedges and transport within wedges. The transport of wedges is geometrical and the transport within wedges can be represented mathematically by a map. As we will show, even without knowing the details of the dynamics within the wedges, it is possible to predict the mixing behaviour to a surprising degree of accuracy.

Consider now the special case of the disk shown in Fig. 1. Transport between wedges occurs only in areas where successive wedges intersect. These intersections take the shape of quadrilaterals whose size varies with fill level, f , defined as the fraction of the diameter occupied by the grains. For uniformly convex containers, analysis immediately reveals the following. (1) For $f < 0.5$, the quadrilateral intersections widen as the fill level

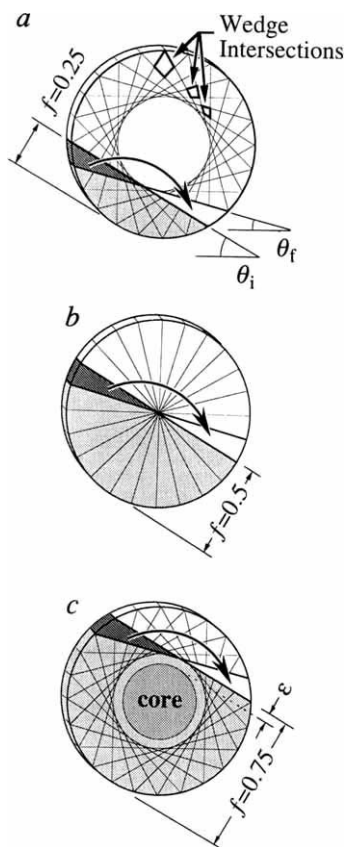


FIG. 1 Schematic of avalanche mixing for a quarter-filled disk (a), a half-filled disk (b) and a three-quarter-filled disk (c). In all cases, the disk rotates slowly clockwise, and distinct avalanches occur which take material from an uphill wedge (dark grey) to a downhill wedge (white), as indicated by solid arrows. Mixing within the wedges is taken to obey a deterministic map; mixing between wedges occurs in quadrilateral wedge intersections indicated.

decreases, so mixing should be faster for lower fill levels. (2) At $f=0.5$, the quadrilaterals collapse (Fig. 1b), and mixing between wedges should vanish. (3) For $f > 0.5 + \epsilon$ (where ϵ is the thickness of a boundary layer, as indicated in Fig. 1c), the wedges cannot penetrate into the centre of the disk, and a non-mixing core should appear. (4) The fractional area of the core should grow as $(2f - 1 - \epsilon/R)^2$, where R is the radius of the disk. (5) Also for $f > 0.5 + \epsilon$, new quadrilaterals emerge, and mixing outside the core should resume. Mixing should be impeded, however, because material must be transported around the core. (6) As the core grows, the distance around the core also grows, and transport (mixing) should again slow. Each prediction is testable and is verified by experiment. In Fig. 2, we show side-by-side comparisons between experiments and numerical simulations, using a random map to model mixing within the wedges. Other maps can be devised to more realistically probe the mixing dynamics—for example using molecular dynamics²⁶, continuum mechanics²⁷ or cellular automata¹ techniques. In our investigations with monodisperse, weakly cohesive granular solids, we find that the simple random map gives surprisingly good agreement.

As an example of a quantitative comparison, we track the positions of the centroids of the two groups of differently coloured particles and normalize the two colour centroid locations. Figure 3a shows typical data as a function of time for one centroid's orbit. Disk rotation causes the centroid orbits to oscillate,

whereas the iterative nature of the avalanches cause the orbits to decay exponentially. The decay time-constant γ , shown in Fig. 3b as a function of f , defines a mixing rate. The mixing rates calculated by simulation, which uses random mixing, are consistently higher than the measured rates for $f < 0.5$. Nonetheless, the experiments and simulations well confirm the geometric predictions.

We can also determine a measure of mixing efficiency and optimum operating point. The volume of material mixed can be written as $V(f)$, where V is the volume of the disk occupied by the granular material, and we take $V(f=1)=1$. Optimal mixing occurs when $\chi \equiv \gamma V(f)$ —the volume of material mixed per characteristic time—is maximized. The inset to Fig. 3b shows the mixing efficiency, with the experimental (calculated) optimum occurring at $f=0.23$ ($f=0.25$). The error bars are due to uncertainties in the exponential fit, which is calculated identically for experimental and computational data.

The model that we have presented is undoubtedly simple. For the future, several extensions are indicated, some of which have been studied in our laboratory. First, we have considered only a quasi-two-dimensional, slowly rotating mixer. Second, we have only presented mixing within uniformly convex containers. Third, we have only discussed monodisperse particles, and fourth, we have neglected interactions between particles (for example, cohesion, triboelectrification and arching) which are known to influence particulate flow.

Extension to three dimensions is not difficult. In three dimensions, slow mixing still occurs via avalanches which effectively transport material downhill from one wedge to another. Wedge shapes vary in a complex way, and wedge maps may be complicated; nevertheless, the same idea holds. We have investigated mixing in more complex geometries as well; for example Fig. 4 shows an experimental/computational comparison of mixing within a thin square container. We have also found that concavities in the container shape (as occur, for example, in a baffled container) can in some circumstances cause the core to be eroded. This occurs because the angle of repose prevents gravity from filling the concavity initially until rotation sufficiently increases the material slope in the cavity. When this secondary avalanche inside the concavity occurs underneath the core, the core location shifts downwards and will thereafter participate in mixing. This complication can be included in simulations in a straightforward way. Segregation effects and particle interactions represent the most serious difficulty in extending our model; nevertheless, such processes can be incorporated into a map-based analysis—for example, we have constructed maps that permit different types of particles to travel different distances. Moreover, experiments with weakly cohesive particles reveal that geometrical structures persist. We hope that approaches of this kind, modelling only the most basic mech-

FIG. 3 a, Centroid position projected onto the material's centreline for an experiment at fill level $f=0.30$. The centroids' positions are taken relative to that of the whole material and normalized to one initially. When the material is perfectly mixed, the colour centroids coincide with each other at the origin. The centroids are calculated from digital pictures taken as the disk rotates. The same algorithm is used to calculate experimental and simulated centroid positions. Dots are experimental data; solid lines are a fit to $\exp(\gamma t) \cos(2\pi t/T)$ with mixing rate $\gamma = 1.1 \pm 0.2$, period $T = 0.37 \pm 0.02$, and time t in units of revolutions. The exponential envelopes are emphasized by dashed lines. b, Mixing rate γ versus fill level f . Open symbols are obtained from fits to simulated data from the model; filled symbols are experiments. For $f > 0.5$ the core is excluded from the calculation of γ . Inset, the volume mixed per characteristic time, χ ($\equiv \gamma V$), versus f . The experimental (calculated) optimum is $f=0.23$ ($f=0.25$).

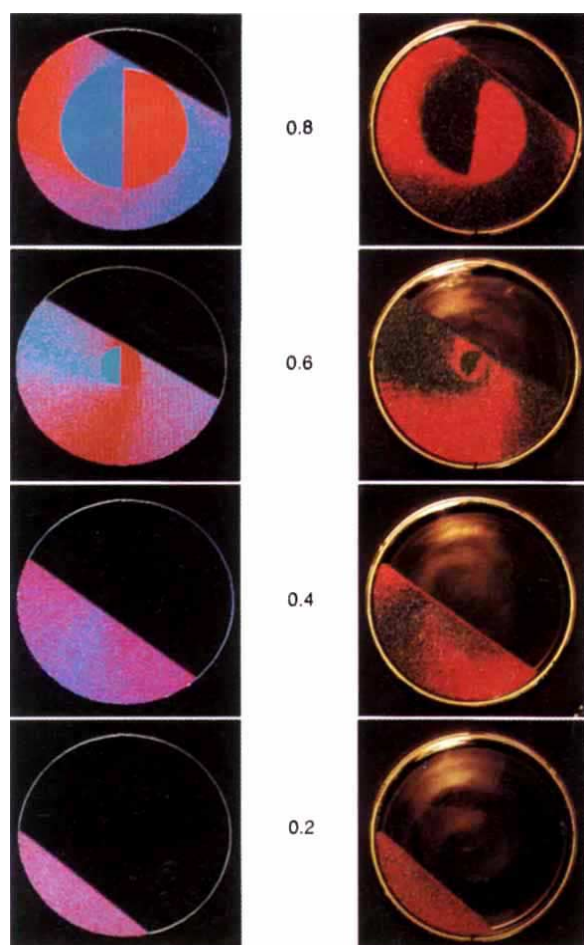


FIG. 2 Mixing patterns from simulation (left) and experiment (right) after two disk revolutions at the indicated fill levels f . To initialize an experiment, we lay a divider along the disk diameter and pour equal amounts of red (blue) salt into the right (left) side. We then remove the divider, seal the top, and set the disk on edge. For low fill levels, note the similar interpenetration of colours across the disk and the equivalent degree of mixedness. For high fill levels, an unmixed core appears with reduced mixing rates outside it.

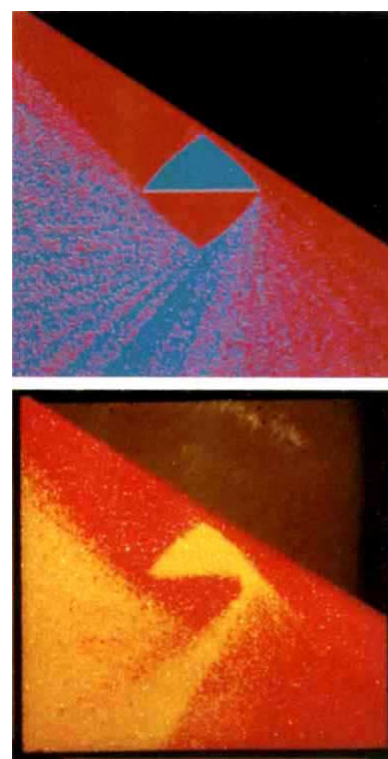
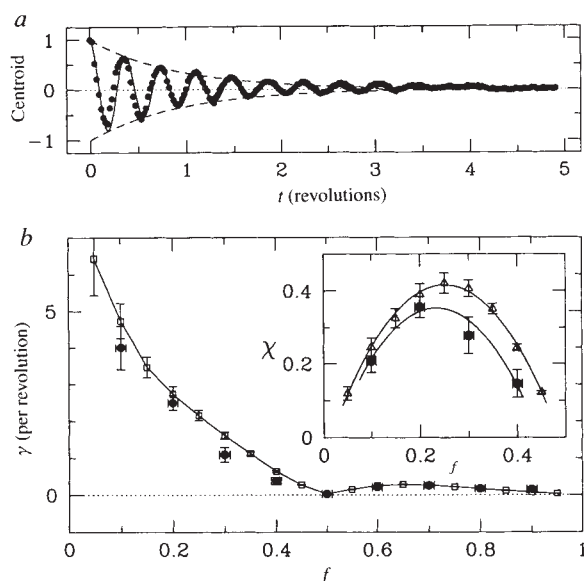


FIG. 4 Granular mixing in a thin square container after 1.25 revolutions. Top, simulation; bottom, experiment. The experiments are initialized as in Fig. 1; here the salt is dyed red and yellow. The simulation here includes a thin layer of thickness $\varepsilon = 10$ grains outside the core to account for the boundary layer mentioned in the text. ε can be measured either directly or through the growth of the core size with f .

anisms and including complications as needs arise, may lead to successful analyses of complex granular flows. $\square$

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Commensurate 'freezing' of alkanes in the channels of a zeolite

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FLUIDS confined in narrow pores can have properties that are distinctly different from those of bulk fluids^{1,2}. Most studies of fluids in pores have focused on simple fluids which can be modelled as near-spherical molecules. But molecular shape can also exert an influence on the fluid's behaviour, particularly for large and/or complex molecules. The adsorption isotherms of alkanes in the zeolite silicalite provide an apparent example of this: the short-chain (C_1 to C_5) and long-chain (C_{10}) alkanes have simple isotherms³⁻⁵ whereas for hexane and heptane the isotherms are kinked^{4,6}, suggesting that some kind of phase transition takes place. Here we present computer simulations of the adsorption of straight-chain hydrocarbons in silicalite, which suggest that this phase transition is of a type not reported previously, arising as a consequence of the interplay between the length of the zig-zag pores and the length of the alkanes. When these two are comparable, the molecules can 'freeze' in a configuration that is commensurate with the pore structure, creating a kink in the isotherms. Such behaviour might be quite general for complex molecular fluids.

Zeolites are crystalline materials that have a well defined microporous structure. Next to their strong acidity, selective adsorption of molecules in the micropores forms the basis of the application of these materials as catalysts in refining and in the petrochemical industries⁷. Traditionally, research has focused on trying to understand catalytic conversion in the pores of the zeolite; less attention has been paid to the sorption properties *per se*. This lack of knowledge has long hampered the understanding of phenomena such as the 'compensation effect', which is the unexpectedly high conversion of long-chain alkanes seen in catalytic cracking⁸. The explanation of this effect has been the subject of many discussions and it is only recently that it has been attributed to differences in sorption behaviour of the alkanes⁸. This example shows that quantifying the sorption effects not only is a prerequisite for the design and optimization of catalysts, but can even lead to a better understanding of the fundamentals of zeolite catalysis.

Adsorption isotherms are also of fundamental interest as they may signal phase transitions, such as capillary condensation or wetting, of the fluid inside the pores². For example, if a system exhibits capillary condensation, one would measure a stepped adsorption isotherm with hysteresis. Steps or kinks without hysteresis are occasionally observed on flat substrates¹. As the pores of most zeolites are of molecular dimensions, adsorbed alkane molecules behave like a one-dimensional fluid. In a one-dimensional system phase transitions do not generally occur⁹ and therefore one would expect that for alkanes the adsorption isotherms are of type I', that is, they do not show kinks or steps. If steps occur, they are usually attributed to capillary condensation in the exterior secondary pore system formed by the space between different crystals³. For silicalite, adsorption isotherms have been determined for various *n*-alkanes: for the short-chain alkanes (methane–pentane) the isotherms are indeed of type I^{3,4}, as observed also for decane^{3,5}. For hexane and heptane, however, a kink or step is observed^{4,6}. To the best of our knowledge, no molecular explanation of this peculiar behaviour of hexane and heptane has been given.

At present, data on sorption properties are scarce because experiments are difficult and time-consuming, in particular at

reaction conditions⁸. Computer simulations seem to be an alternative to quantify the sorption behaviour. Molecular simulations of adsorption are conveniently performed in a grand-canonical ensemble, which corresponds closely to the experimental arrangement: a zeolite in open contact with a reservoir that fixes the temperature and chemical potential. The number of particles in the zeolite is allowed to fluctuate via Monte Carlo moves in which one attempts to add or remove a particle. Particularly

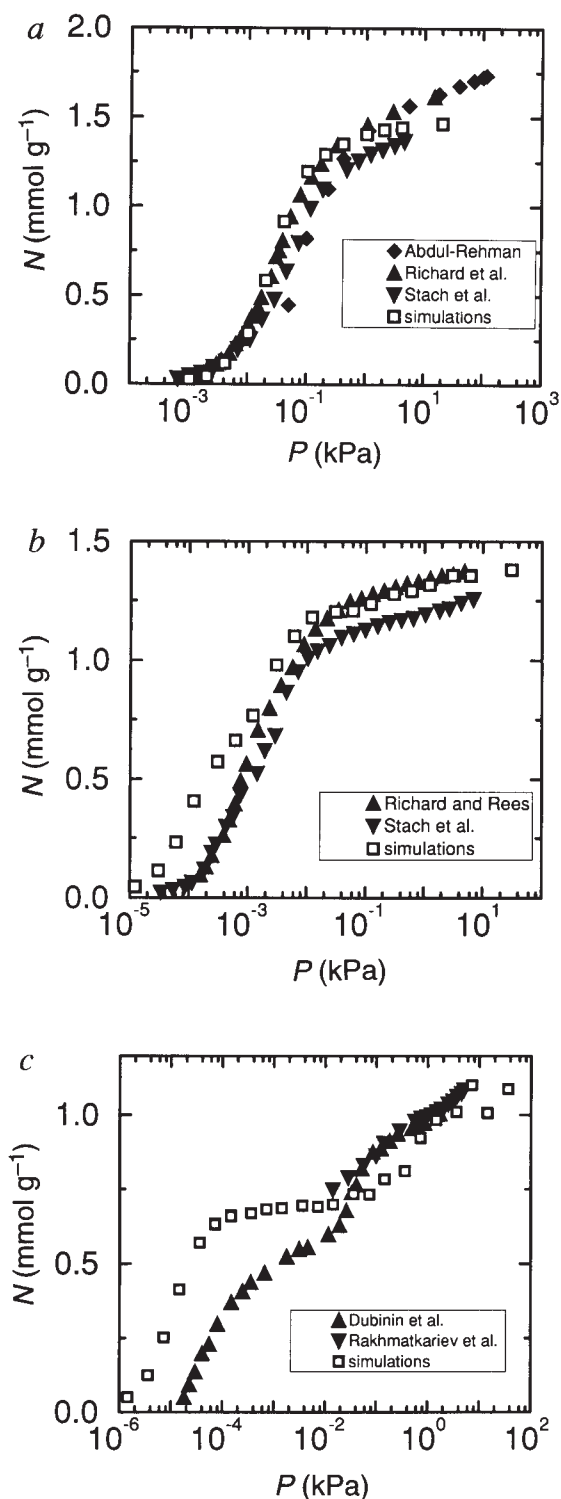


FIG. 1 Adsorption isotherms of butane (a), hexane (b) and heptane (c) in silicalite. Closed symbols, experimental data^{3,4,6,18,19}; open symbols, results of simulations for temperature $T = 298$ K (this work). P , pressure of gas; N , mmol of gas absorbed per g silicalite.

for chain molecules, the insertion of molecules tends to be a disfavoured process in a conventional grand-canonical simulation¹⁰. To see this, consider a simulation of methane molecules adsorbed in a zeolite. Assume that a methane molecule can be inserted successfully if this molecule is placed at a randomly selected position where it does not overlap with one of the other atoms of the system. Depending on the loading, one needs of the order of 10^3 attempts to find such a position, for ethane this is of the order of 10^6 , and for heptane even 10^{21} . The latter would result in a simulation of many years on a supercomputer, and allowing such simulations to equilibrate would be prohibitively time-consuming. For this reason, simulations of adsorption have focused on systems containing small molecules. To make grand-canonical simulations of long-chain alkanes possible, we have used the configurational-bias Monte Carlo technique⁹⁻¹⁴ for the insertion step. In this technique a molecule is inserted atom by atom in such a way that overlaps are avoided. This 'growing' procedure introduces a bias that can be removed exactly by appropriate acceptance rules¹⁵. Depending on the chain length and conditions, this technique can be 10 to 50 orders of magnitude more efficient than a conventional grand-canonical simulation.

Here we model alkane molecules using a 'united-atom' model in which CH_3 and CH_2 groups are considered as single interaction centres¹⁶. The zeolite is modelled as a rigid crystal and the zeolite-alkane interactions are assumed to be dominated by dispersive interaction with the oxygen atoms, which are described

with a Lennard-Jones potential: $u(r) = 4\epsilon[(\sigma/r)^{12} - (\sigma/r)^6]$. The size (σ) and energy parameters (ϵ) have been fitted to the heats of adsorption and the Henry coefficients of the *n*-alkanes ranging from butane to decane in silicalite: $\epsilon_{\text{CH}_3} = 87.5$ K, $\epsilon_{\text{CH}_2} = 54.4$ K and $\sigma_{\text{CH}_3} = \sigma_{\text{CH}_2} = 3.64$ Å. The scatter in the experimental data for the Henry coefficients makes it difficult to arrive at a unique set of parameters. The values used in this work give a reasonable overall description of the Henry coefficients of these alkanes, but turned out to be less optimum for hexane and heptane (B.S., manuscript in preparation). In the simulations we used periodic boundary conditions and the simulation box contained 16 unit cells of silicalite with total size $40.14 \times 39.840 \times 53.680$ Å³. The number of trial orientations in the configuration-bias Monte Carlo moves ranged from five for butane to eight for heptane¹⁴.

In Fig. 1 the simulated adsorption isotherms of various alkanes in silicalite are compared with experimental data. For butane a type I isotherm is observed, and the agreement between experiments and simulation is good. For hexane and heptane there is some agreement at high pressures, but at low pressures deviations exist which indicate that the zeolite-alkane model may be further improved. It is interesting to note that for heptane both the experiments and the simulations show a step at approximately half the loading. Also for hexane detailed inspection of the calculated adsorption isotherm shows a kink at this loading. As the simulations are performed on a perfect single crystal, these deviations from the type I isotherm must be due to a transition of the fluid inside the pores and can not be attributed to the secondary pore system.

Silicalite has two types of channels, straight and zig-zag, which are connected via intersections. Figure 2 shows 'snapshots' of hexane molecules in the channels of silicalite at two different loadings: Fig. 2a is at approximately half the maximum loading and in Fig. 2b the zeolite is almost saturated. Note that the length of a hexane molecule is of the order of the length of the period of the zig-zag channel. At low chemical potential, the hexane molecules move 'freely' in these channels and the molecules will be in the intersections for some of the time. If part of the intersection is occupied, other molecules can not reside in the straight channels at the same time. At high pressures, almost all hexane molecules fit exactly into the zig-zag channel (Fig. 2b), and we find that they no longer move freely. In such a configuration the entire straight channel can be tightly packed with hexane molecules. This may explain the plateau in the adsorption isotherm: in order to fill the entire zeolite structure neatly, the hexane molecules located in zig-zag channels have first to be 'frozen' in these channels. This 'freezing' of the positions of the hexane molecules implies a loss of entropy, and will

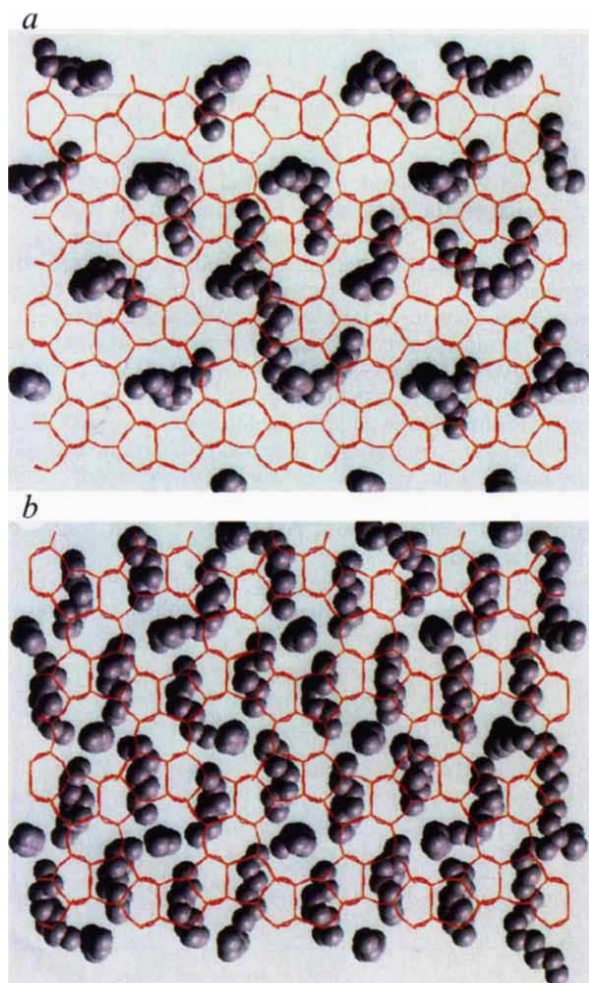


FIG. 2 Hexane in silicalite: a, at approximately half the maximum loading; b, at almost maximum loading. The zig-zag channels are in the plane of the page, straight channels are perpendicular to the page. The hexane molecules are represented by grey spheres and red/orange lines represent the zeolite framework.

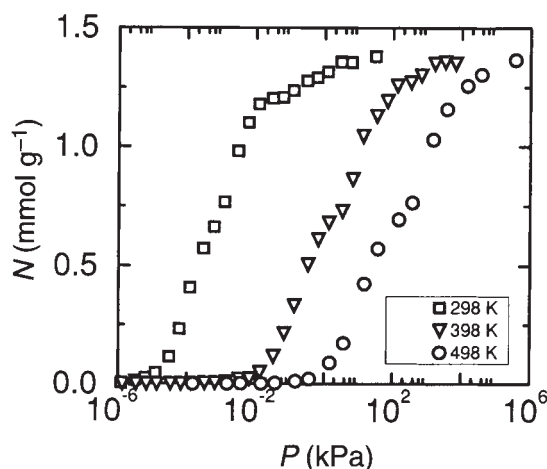


FIG. 3 Temperature dependence of the adsorption isotherms of hexane as obtained from simulations.

therefore only occur if the pressure (or chemical potential) is sufficiently high to compensate for this loss. This also makes clear why we do not observe a step for molecules that are shorter or longer than hexane or heptane. If the molecules are longer, they will always be partly in the intersection and nothing can be gained by a collective 'freezing' in the zig-zag channels. If the molecules are shorter than one period of the zig-zag channel, a single molecule will not occupy an entire period and a second molecule will enter which results in a different type of packing.

We can use these results to shed some light on the apparently conflicting data for hexane: the adsorption isotherms obtained by Lohse *et al.*¹⁷ do not provide evidence for a kink at half loading whereas the one measured by Richard and Rees⁴ does show such a kink. One difference between these sets of data is that the former were measured at temperature $T=298$ K, whereas the latter were measured at $T=308$ K. For higher temperatures, Richard and Rees observed that this kink becomes more pronounced. Figure 3 shows that the kink also becomes more pronounced in the simulations, and with increasing temperature even grow out to a small plateau. At high temperatures the entropy contribution is more important and a higher pressure must be imposed before the hexane molecules 'freeze' or get locked in the zig-zag channel; apparently the temperature in the experiments of Lohse *et al.*¹⁷ was just too low to observe a kink. As far as we are aware, such a transition has never been observed experimentally. It is interesting to note that the observed 'freezing' is similar to the commensurate-incommensurate transitions observed for noble gases on graphite (ref. 20, and references

therein). In both cases the geometrical aspects of the substrate play an essential role in 'locking' the fluid into a specific structure. □

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Controlled growth of microporous crystals nucleated in reverse micelles

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THE development of new methods for nucleating and growing microporous crystals has made available new framework structures and morphologies for these technologically important materials¹. These approaches have included solution-based synthesis² and the use of simple organic structure-directing agents³ and complex organic assemblies such as liquid-crystal phases^{4,5}. Here we show that the growth of microporous zincophosphate^{6–8} with the sodalite structure can be controlled by preparing the crystals from reactants included within the interior aqueous phase of reverse micelles dispersed in an organic solvent. The growth of inorganic phases in reverse micelles has been exploited previously for the preparation of monodisperse oxide⁹, semiconductor¹⁰ and metal particles¹¹. In this study, we introduce the two inorganic components—zinc and phosphate ions—in separate micelles, so that crystallization is controlled by the collision and exchange kinetics of the surfactant structures. Moreover, the surfactant–water interface provides the site for crystal nucleation, favouring initial nucleation at the (111) and/or (110) crystal faces and subsequent growth of the zincophosphate crystals by deposition along the {100} faces. The growth process is ultimately interrupted by sedimentation when the crystals grow large enough, producing a precipitate of microcrystals several hundred nanometres in size. Our results indicate that this approach can provide a means of controlling the morphology as well as the size of growing crystals.

Mann and Williams reported that the crystallization of Ag_2O along specific crystal faces could be controlled by migration of OH^- through a Ag^+ -containing vesicle interface¹². Our goal was to develop a similar strategy for controlled crystal growth of microporous materials. Reverse micelles exist in certain compositions of oil–water–surfactant within a well defined temperature range¹³. The most commonly used surfactant in making reverse micelles is AOT (12 bis(2-ethyl hexyloxy)carbonyl)-1-ethane sulphonate), with *n*-alkanes as the oil phase¹³. At temperatures used for conventional aluminosilicate zeolite synthesis ($>90^\circ\text{C}$), the reverse micellar structure is unstable and results in phase separation^{14,15}. Thus we chose to work with the zincophosphate system, which Stucky and co-workers recently discovered can be synthesized under ambient conditions^{6–8}, with frameworks isostructural with zeolites.

The experimental strategy involved making a reverse micelle containing zinc ions and another with phosphate ions and then

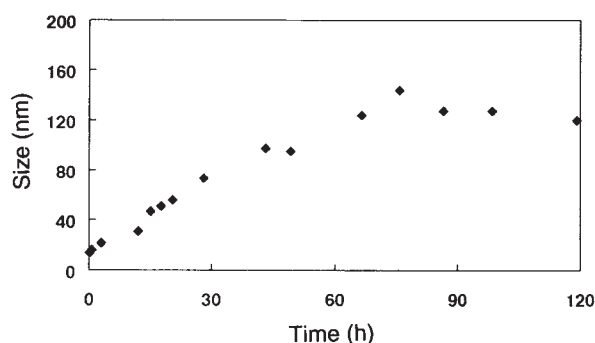


FIG. 1 Particle growth during synthesis of zincophosphate sodalite measured by dynamic light scattering. The initial size of the zinc-containing and phosphate-containing micelles were 8 and 15 nm, respectively.

mixing these solutions to form the microporous material. Several concentration ranges of AOT (0.065–1 M), zinc solution (0.07–0.3 M) and phosphate solution (0–1.25 M tetramethylammonium hydroxide) were investigated. Reactions were carried out at various times after the micelles were formed, at reaction temperatures of 20–40 °C. Over these ranges, hopeite, the hexagonal phase $P6_1$, sodalite and amorphous phases were obtained, typical for this system⁸. Use of a non-ionic surfactant Igepal resulted in microemulsion formation rather than reverse micelles. Below we outline the procedure for formation of sodalite. AOT was purified and dissolved in *n*-hexane to make a 0.0675 M solution. An aqueous solution of $Zn(NO_3)_2 \cdot 6H_2O$ with a concentration of 0.2 M was prepared. Another aqueous solution containing 0.50 M H_3PO_4 , 0.25 M NaOH and 1.05 M tetramethylammonium hydroxide was also prepared. A quantity (2 ml) of each of these aqueous solutions was mixed with 50 ml of the AOT/hexane solution, gently shaken for a minute, and then left undisturbed for 48 h. The excess aqueous phase (~1.1 ml for zinc and 0.5 ml for phosphate) was removed from each solution, and analysed to determine the uptake of the species in the micelles. For the zinc micelle, the $[AOT]/[H_2O]$ ratio was 13 and the concentration of zinc in the reverse micelle was 0.0075 M. In the phosphate-containing micelle, the $[AOT]/[H_2O]$ ratio was 21 and the phosphate concentration was 0.0125 M. Tetramethylammonium ion was required for the

uptake of the phosphate into the reverse micelle and was not incorporated into the sodalite framework. Within 7–10 days after removing the aqueous phase, 40 ml of the zinc-micelle solution was mixed with 50 ml of the phosphate-micelle solution at room temperature to form zincophosphate sodalite.

We used dynamic light scattering to measure the particle size as a function of time (Fig. 1). The particles grow in size in a linear fashion from 14 nm to ~150 nm over a period of three days and then remain stable around ~140 nm. The solution remains optically clear and a white solid is found at the bottom of the reaction vessel after four days, its amount gradually increasing with time. The samples that were recovered after four days of reaction at the first visible sight of settled crystals were examined by X-ray powder diffraction and electron microscopy. As the samples collected were minute, they were examined without washing off the surfactant. Figure 2a shows the diffraction pattern of this sample. The reflections due to the zincophosphate sodalite framework are present in the powder pattern along with reflections characteristic of the surfactant covering the crystals. The weakness and the low signal-to-noise ratio arise from the limited sample size. Figure 2b shows the diffraction pattern of methanol-washed samples recovered after two weeks of reaction, when it appeared that no further crystals were forming. The morphology of the crystals was obtained from the scanning electron micrographs, shown in Fig. 3. The samples recovered after four days exhibit triangular faces, with a size of ~600 nm. The samples recovered after two weeks also are primarily of the same morphology, though cubic crystals are also clearly evident. The size distribution is smaller, with crystals more typically between 400–500 nm.

There are several aspects to this crystal growth process that are different than the conventional process. The original zinc and phosphate aqueous solutions used above to make the micelles form an amorphous precipitate on mixing (pH 11.3) which does not transform to a crystalline form. Introducing the

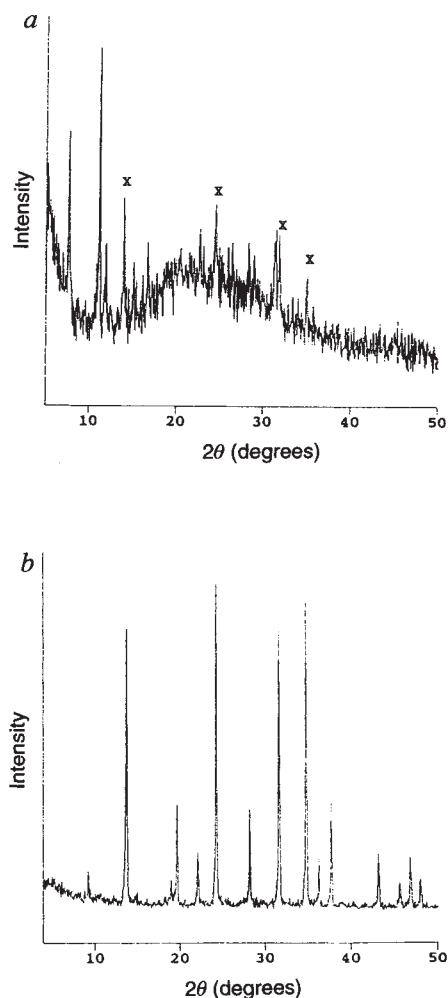


FIG. 2 Powder X-ray diffraction patterns of material recovered from the reactor system after: a, four days (x marks the sodalite reflections; the reflections at 7.5°, 10.7° and 11.5° are from AOT on the crystal surface); b, 14 days (AOT was washed out by methanol).

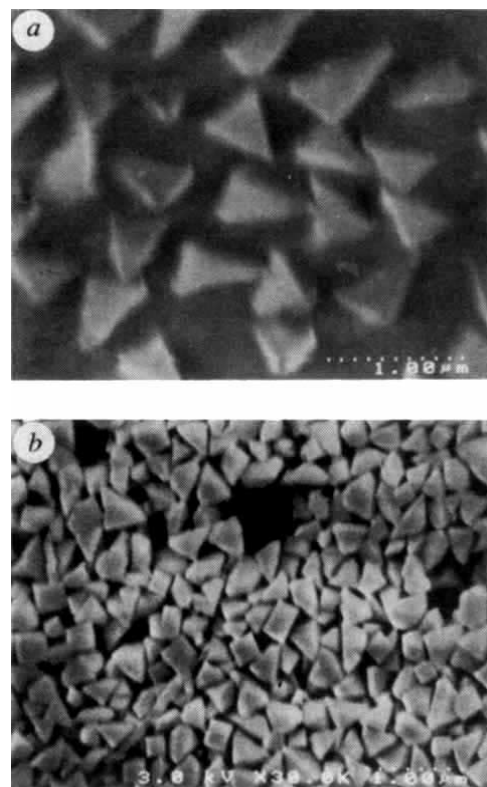


FIG. 3 Scanning electron micrographs of crystals recovered after 4 days (a) and 14 days (b). Scale bars (dotted): top, 1.0 μm; bottom, 1.0 μm.

reactants via reverse micelles results in a different intramicellar composition than the starting solutions. Gier and Stucky's reported procedure necessitated a pH of 7.4 and proceeded through an amorphous gel into sodalite⁶, whereas in the micellar system, there was no immediate formation of an amorphous phase. The reverse micelles are clearly influencing the mixing of the reactants. These micelles diffuse by brownian motion and collisions between them can result in fusion. The time period of existence of dimers is of the order of microseconds¹⁶. This temporary fusion causes incorporation of zinc and phosphate species in the same micelle, and reactions ensue between them leading to nucleation of the sodalite crystals. The particle-growth data show continuous linear growth, unlike the sigmoid curves observed during nucleation in conventional microporous-material synthesis. The surfactant-surrounded nuclei grow into crystals by incorporating species from other micelles via collisions promoted by diffusion and convection. The crystals remain suspended as long as the thermal brownian diffusive motion balances the gravitational forces. For the 600-nm crystals, we calculate from the Stokes relation¹⁷ a terminal settling velocity of 0.5 cm h^{-1} , which would result in particles settling out in a maximum of 10 hours. Once settled, the crystals lose contact with the nutrient pool and stop growing. Recent work on crystallization of silicalite from clear solutions showed that if the product is removed from the system, nucleation and crystal growth will restart¹⁸. In our case, sedimentation effectively stops further growth of settled crystals, whereas in solution nucleation and crystal growth continue. This leads to an equilibrated steady-state value of 140 nm for the size of the suspended crystallites. The smaller sizes of the settled crystals recovered after 14 days of reaction (400–500 nm) as compared to the crystals recovered after 4 days (600 nm) arise owing to increasing number of crystals being nucleated with time, resulting in competition and depletion of the nutrient pool.

The most interesting aspect is the morphology of the crystals (Fig. 3). Sodalites typically exhibit morphologies characteristic of their cubic symmetry, which are manifested as cubes and octahedra^{19,20}. Several cubes are seen in Fig. 3b. Of more interest, however, are the incomplete cubic crystals seen in Fig. 3a and b, which exhibit triangular faces, and can be viewed as partially formed cubes, with the (111) or (110) faces stabilized. A diagram of such structures and their connection to the cube is shown in Fig. 4. Crystal growth rates can be strongly influenced by the adsorption of "impurities" on a crystal face²¹. In the present case, as a single {111} and/or {110} face is being manifested, these faces are possibly the sites for preferential binding of the surfactants, and form the original template for nucleation. Thus the sulphonate groups of the AOT at the interface of the reverse micelle align the Zn^{2+} ions along the (111) and/or (110) face that begins the nucleation process. This leads to crystal growth in a layer-by-layer fashion on the {100} faces. It is interesting to note that in a recent study on another zincophosphate crystal of cubic morphology, the crystals exhibited a preferential growth along the (111) face on a zirconium phosphonate substrate²².

In previous experiments using reverse micelles and vesicles for synthesis of solids, the particle growth was primarily a precipita-

tion process due to supersaturation^{13,23}. In the work reported here, the reverse micelles help nucleate the crystals, and micellar interactions enable crystal growth by deposition along specific crystal faces. Evaluation of the features on the growing crystal face as a function of time will provide a better understanding of the mechanism of crystal growth of microporous materials. □

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Variations in atmospheric methane concentration during the Holocene epoch

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RECORDS of the variation in atmospheric methane concentration have been obtained from ice cores for the past 1,000 years and for the period 8,000–220,000 yr BP (refs 1–4), but data for the intervening period, spanning most of the present interglacial period (Holocene), are patchy (refs 5–7 and references therein). Here we present a continuous, high-resolution record of atmospheric methane from 8,000 to 1,000 yr BP, from the GRIP ice core in central Greenland. Unlike most other climate proxies from ice cores (such as oxygen isotope composition⁸ and electrical conductivity⁹), methane concentrations show significant variations—up to 15%—during the Holocene. We have proposed¹ that variations in the hydrological cycle at low latitudes are the dominant control on past levels of atmospheric methane. This is now supported by the observation that the lowest methane concentrations in our new record occur in the mid-Holocene, when many tropical lakes dried up¹⁰. The concentration increases during the Late Holocene, probably owing to an increasing contribution from northern wetlands.

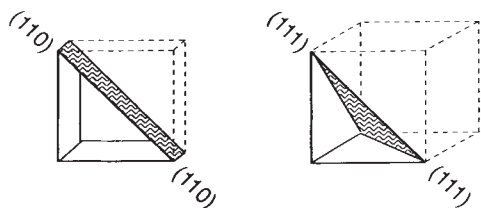


FIG. 4 A schematic presentation of crystal growth of the zincophosphate sodalite by nucleation along the (110) or (111) faces.

Earlier reconstructions of past CH_4 changes from both Greenland and Antarctic ice cores^{1,5,7} showed that glacial-interglacial climate changes are accompanied by large variations in atmospheric methane concentration. Recently, detailed results¹ from the GRIP core (Summit, central Greenland; 72° 34' N, 37° 37' W; altitude, 3,230 m) revealed that rapid variations in Greenland climate during the last glaciation and deglaciation⁸ are reflected in the methane record. Over the past millennium, measurements on the Eurocore ice core (Summit) showed that the CH_4 pre-industrial level fluctuated by ~10%. Although these fluctuations are only partially correlated with climate variations², it was concluded that the large variations during and at the end of the last glaciation were mainly linked to changes in the hydrological cycle at low latitudes¹. Thus atmospheric CH_4 concentration changes in the Holocene epoch are expected to parallel (at least to a certain degree) large-scale climate changes. In particular, it was suggested¹⁰ that there could be another distinct CH_4 decrease in the Middle Holocene when many tropical lakes dried up.

So far, the Holocene has been investigated in detail only for the past millennium^{2,4}. For the earlier Holocene, less detailed measurements have been made on various ice cores (refs 5–7 and references therein), but either the scatter of the results was too large or the number of the measurements along one single core too small to allow for a detailed reconstruction of the atmospheric CH_4 concentration. The Early Holocene part of the GRIP core originates partially from a zone of brittle ice. This demands special precautions for the preparation of samples which are fulfilled by allowing the core one year of relaxation and by selecting unbroken core pieces.

The measurements presented have been performed in Bern with a dry extraction technique² and in Grenoble with a melting-refreezing method¹. In both laboratories the extracted air is analysed by gas chromatography. The analytical precision amounts to ± 37 and ± 20 (2 σ) parts per billion by volume (p.p.b.v.) for Grenoble and Bern, respectively^{1,2}. Replicate measurements on the same depth levels fit into the estimated analytical precision.

Grenoble measurements give values that are generally ~30 p.p.b.v. lower than those obtained in Bern. Standard-gas intercalibration between the laboratories has shown no difference, but extraction methods have been slightly changed in both laboratories since coherent intercalibrations were obtained². The difference between values obtained by the two laboratories probably reflects the uncertainties in estimating the contamination introduced by the extraction systems, as well as potential fractionation effects during transfer of the gas samples through the extraction lines. In any case, the profiles from the two establishments reveal very similar trends, suggesting a rather stable systematic shift. We homogenized the data sets so that the mean values of overlapping parts agree (constant shift of +15 and -15 p.p.b.v. for Grenoble and Bern, respectively). This rather arbitrary correction does not affect the CH_4 Holocene trends depicted by our record. The new measurements agree within the error limits with our previous measurements from Eurocore².

The timescale for the ice is provided by the GRIP glaciological model⁸, modified such that the best fit on Holocene stratigraphic markers is obtained (S. Johnsen, personal communication). At Summit, the air-enclosure process takes place ~70 m below the surface under present-day conditions. Thus the air in the bubbles is younger than the surrounding ice. Today's age difference amounts to¹¹ ~210 yr. Because of the diffusive mixing in the firn and the gradual enclosure process, the air in the bubbles has not a discrete age but rather an age distribution with a standard deviation of ~7 yr. Climate conditions over the Holocene at the GRIP site did not vary significantly⁸. Therefore the past air-ice difference in age, calculated with a semi-empirical model of firn densification¹², varies little (between 200 and 240 yr) for the depth levels presented here.

Samples from a total of 92 depth levels were analysed. The samples were distributed alternately between the two laboratories. The mean difference between depth levels is 13 m (minimum 3 m, maximum 36 m) corresponding to a mean time resolution of 85 yr (range from 29 to 228 yr).

The atmospheric methane concentration is plotted against age and depth together with the GRIP isotopic record⁸ in Fig. 1. The trends depicted by the two laboratories agree remarkably well except in the time interval 7–8 kyr BP, corresponding to 1,220–1,320 m depth, where the results from Bern are (after the +15/-15 p.p.b.v. correction) up to 50 p.p.b.v. higher than the Grenoble ones, combined with unusually high scattering. Some ice samples from this interval have been exchanged between the laboratories for re-measurement. We find again that the results from Bern are unusually high compared to results from Grenoble. We note that this effect occurs in a depth where air bubbles end their transformation into air-hydrates, due to the increasing pressure with depth (S. Kipfstuhl, personal communi-

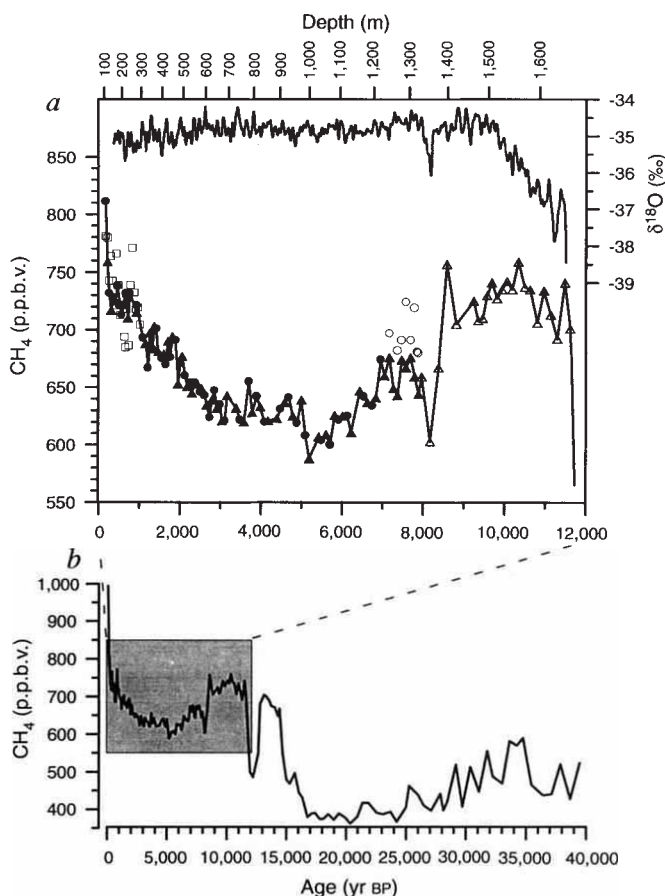


FIG. 1 a, Mean atmospheric methane concentrations from the Greenland ice-cores GRIP (ref. 1 and this work) and Eurocore² over the past 40,000 yr (mean values for each depth level). Note that the plot ends at 1,000 p.p.b.v., whereas today's concentration is ~1,700 p.p.b.v.; the spike at 800 yr BP originates from the more detailed Eurocore record. b, Expanded-scale plot of data for the past 12,000 yr. Top trace, isotopic record⁸ smoothed by a 11-m gaussian low-pass filter based on 0.55-m data sets. Bottom trace, methane record: filled circles and filled triangles mean results from Bern and Grenoble respectively (this work); open circles, measurements affected by air-hydrates (see text); open triangles, results from ref. 1; open squares, mean values from the Eurocore record². Note that the timescale is in calendar years; Bern results are lowered by 15 p.p.b.v., whereas results from Grenoble and ref. 1 are raised by 15 p.p.b.v., to homogenize the records.

cation). As the dry extraction used in Bern leads to higher concentrations than the Grenoble wet extraction, this suggests that at these depths, the bubbles, easily opened by the dry extraction, contain an over-proportional amount of methane versus nitrogen and oxygen, and that a significant part of air-hydrates resist the crushing. This hypothesis is supported by the observation of decreasing efficiency (by $\sim 10\%$) of the dry-extraction process in the corresponding depth range. Unfortunately, the dry-extraction efficiency cannot be quantified accurately enough to allow a correction to the measured concentrations. We therefore consider in this depth range only the results of the wet extraction, where most of the gas stored in bubbles and air-hydrates is obtained, as a signal of the atmospheric CH_4 concentration.

The most striking feature of the CH_4 profile plotted in Fig. 1 is the observation of significant variations throughout the Holocene epoch. The highest concentrations are found during the first 2,000 yr of the interglacial, and then not before the past millennium; during the Middle Holocene, concentrations are lower by as much as 100 p.p.b.v.. Such general features of the CH_4 record have no analogy in the GRIP climate isotopic record. In addition, over most of the Holocene, the background CH_4 level stands more around 650 p.p.b.v. than at the commonly accepted 700 p.p.b.v. interglacial level (which is mainly based on concentration measurements from the recent millennium). Our data is in accordance with the trends noted in previously published data⁵⁻⁷. However, the uncertainty in dating and the scatter of published records precludes a detailed comparison.

Going forward in time, from 11.5 to 8.5 kyr BP, CH_4 concentrations fluctuate slightly around a mean 725-p.p.b.v. level; then a sharp decrease to 600 p.p.b.v. happens¹ at 8.2 kyr BP, followed by an increase towards a mean level of 670 p.p.b.v.. Between 7 and 5.2 kyr BP, methane decreases again and reaches its lowest Holocene concentration. The following increase occurs in steps. A first rapid increase of 40 p.p.b.v. takes place within ~ 200 yr. During the following 2 kyr, concentrations stay constant at ~ 630 p.p.b.v.. This plateau is followed by an increase until $\sim 1,000$ yr BP, where methane reaches its Greenland pre-industrial 730-p.p.b.v. level, as found in other Greenland records^{2,4}.

The abrupt fall and subsequent rise in methane at 8.2 kyr is paralleled by a similar feature in the isotope record. The two signals are synchronous within our time resolution, that is, ~ 100 yr. As the CH_4 oscillation has its origin in large-scale climate shifts, whereas the isotope signal is of a more regional character, a correlation between the two signals indicates that the climate event at 8.2 kyr BP affected a significant part of at least the Northern Hemisphere.

We discussed previously¹ the potential mechanisms driving past natural CH_4 changes, in particular during the sequence of the last deglaciation. Three potential scenarios could explain the glacial-interglacial CH_4 doubling: clathrate outgassing, decrease of the tropospheric oxidative capacity, and increase in global wetland extent. We favoured the large extension of low-latitude wetlands, depicted by other palaeoclimate data as the most probable scenario. We still exclude the first two scenarios as the main explanations of the Holocene CH_4 variations, as discussed below.

First, clathrate outgassing: the Early Holocene was the time of the large retreat of the Laurentide ice sheet (until 7 kyr BP), which should have led, according to the clathrate hypothesis¹³, to a large transient increase in the methane concentration from catastrophic burst events potentially occurring in permafrost regions and/or in continental shelves. Our detailed profile shows no evidence for the occurrence of this kind of event. Such absence cannot be due to the air-trapping process, which could only smooth variations of less than ~ 20 yr duration under Holocene conditions. However, we do not rule out continuous clathrate decomposition (from melting permafrost) as a methane source after the deglaciation. But the weak temperature variations together with source estimates for a global warming

scenario¹⁴ suggest that this source of CH_4 was relatively small in the later Holocene.

Second, changes in the oxidative capacity of the atmosphere: there is a consensus that changes in the OH concentration were not the driving factor for the CH_4 increase, either from the Last Glacial Maximum to the pre-industrial Holocene, or from the pre-industrial Holocene to today¹⁵. Because the Holocene is, on a global average, a stable climate period, we assume that parameters expected to affect the OH level independently from CH_4 itself (NO_x , CO) stayed fairly constant and were thus not a primary factor for changes in the methane concentration.

We cannot exclude the possibility that changes in soil uptake of methane may have exhibited a minor effect on atmospheric methane during the Holocene. But soil sink is an order of magnitude smaller than emissions from wetlands¹⁶, so we feel that any effect of this sort would be negligible. In view of these considerations, and for the reasons below, we conclude that the Holocene methane record presented here reinforces the idea that low-latitude wetlands exert the main controlling influence on atmospheric methane.

Conditions were not suitable for wetland formation after the deglaciation in today's main source regions¹⁷ at high northern latitudes. Western Canada¹⁸ and Alaska¹⁹, as well as Scandinavia²⁰ (until around 8 kyr BP), were generally dry during the Early Holocene; in central Canada²¹, major peat formation started only after 7 kyr BP. (Note that all the dates of palaeodata given here are corrected to calendar years). In contrast, observations from various regions, (including the Nile delta²², India²³, Brazil²⁴, the Sahel and Sahara, Tibet and China²⁵) suggest that at low latitudes, the Early Holocene period was the wettest time of the present interglacial.

The episode of low methane concentration at 8.2 kyr coincides with an episode of drought in tropical Africa²⁶⁻²⁸ and Tibet²⁹. The drought episode around 8.2 kyr BP might have been even more severe than during the Younger Dryas in some regions²⁶. The effect on atmospheric methane is possibly counterbalanced to a certain extent by an expansion of wetlands in the Russian plain³⁰ and Scandinavia³¹.

Low-latitude climate became drier¹⁰ after 5.7 kyr BP. The CH_4 concentration minimum between 5.7 and 5.2 kyr BP is consistent with the maximum aridity at low latitudes¹⁰.

We now consider the methane trends since 5.2 kyr BP. The relative role of low- and high-latitude wetlands seems to have changed since this time; indeed, low latitudes experienced increasingly arid conditions, particularly²⁵ since 3 kyr BP. On the other hand, the peat growth rate in Canada²¹ and Sweden³¹, as well as humidity in Alaska¹⁹, significantly increased from 3 to 1 kyr BP. Furthermore, high peat formation rates are reported for the period between 5 and 1 kyr BP in the Fore-Ural³⁰. Such Late Holocene peat development in today's biggest wetlands is a good candidate for the cause of the CH_4 concentration increase observed at that time. Overall, methane is probably a good indicator for the global wetland extent; even so, the concentration information by itself does not allow us to quantify the relative importance of high- and low-latitude wetlands. Such quantification could be achieved by performing a similar study on Antarctic ice, using the Greenland/Antarctic CH_4 concentration difference³² which is directly related to the high-/low-latitude methane emission ratio. The well defined CH_4 variations during the Holocene will also be useful for temporal correlation of the Holocene parts of ice cores. $\square$

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Evidence for Tibetan plateau uplift before 14 Myr ago from a new minimum age for east–west extension

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IMPORTANT changes in South Asian climate occurred in the Late Miocene epoch (~8 Myr ago)^{1,2}, and these have been attributed by some researchers to uplift of the Tibetan plateau at about the same time^{3–5}. Unfortunately, this link has been difficult to test because the timing of plateau uplift remains poorly constrained by independent evidence. One way to determine the minimum age of uplift is to establish the initiation age of the north-striking normal fault systems in southern Tibet that are widely regarded^{6–10} as being related to gravitational collapse of the Tibetan plateau. Here we report an ⁴⁰Ar/³⁹Ar age of ~14 Myr for hydrothermal mica from an extensional fracture belonging to such a fault system in north-central Nepal. This age implies that east–west extension began before ~14 Myr ago in at least some parts of the Tibetan plateau, suggesting that the plateau attained its high mean elevation well before Late Miocene time.

The tectonic evolution of the Himalayas and Tibet since the Palaeogene collision between India and Asia has been controlled by three classes of deformational structures. The most obvious features are east-striking, north-dipping thrust fault systems and subordinate folds related to shortening and crustal thickening. Some of these, such as the Neogene Main Central and Main Boundary thrust systems, have been traced for hundreds of kilometres parallel to the strike of the orogen (Fig. 1)^{11,12}. The second class of structures includes east-striking, north-dipping normal faults of the South Tibetan detachment system¹³. Developed near the crest of the Himalayas along the southern margin of the Tibetan plateau, these Late Oligocene–Pliocene extensional

structures are thought to have moved in concert with contractional faults such as those of the Main Central thrust system in order to moderate high topographical and crustal-thickness gradients arising from the India–Asia convergence^{14,15}. Active tectonics of the southern Tibetan plateau are characterized by east–west extension on a third class of structures: north-striking, east- and west-dipping normal faults and related strike-slip features (Fig. 1)^{6,7}. Based on the results of numerical experiments on the dynamics of continental plateau uplift, there is general agreement that east–west extension in Tibet was triggered by elevation of the plateau to the point when topography-related extensional stresses exceeded compressional stresses related to continent–continent collision^{5,6–10}.

Most estimates of the time at which east–west extension began, and thus the minimum age of the plateau attaining its high mean elevation if conventional wisdom proves correct, have been based on chronostratigraphic data for extensional basin deposits related to the second and third classes of structures near the southern margin of the plateau. The most extensively studied Neogene sedimentary sequence in this area is found in the Thakkhola and Ghyrong grabens (Fig. 1). Both are characterized by a lower section of fluvial and lacustrine strata separated from an upper fanglomerate section by an angular unconformity^{16,17}. Palaeontological and palaeomagnetic data suggest that units found beneath the unconformities range in age from Late Miocene to Early Pliocene^{18–20}. The structural setting of the basins in which these rocks were deposited is poorly understood, but the basal units found within the Thakkhola graben overlap NNE-striking extensional faults¹⁷, suggesting that east–west extension may have started before Late Miocene basin sedimentation. There is no question that units above the unconformity were deposited synchronously with the opening of north-trending grabens^{17,18}. Palaeomagnetic investigations of these strata indicate Late Pliocene and younger ages. The lack of ambiguity associated with assigning a structural setting to these deposits prompted some researchers^{8,18} to postulate a 5–2 Myr age for the inception of east–west extension. More recently, structural and geochronological studies along the western flank of the Yangbajian graben (Fig. 1) suggest that east–west extension in at least one part of the plateau began 11–5 Myr ago^{21,22}.

Our new constraints on the age of this phase of extension are derived from current studies of the structural evolution of the Annapurna range of north-central Nepal, east of the Thakkhola graben (Fig. 2). The extensional faults that are overlapped by basal units of the Thakkhola Neogene sequence are part of a regionally important family of NNE-striking normal faults with minor displacements that disrupt Palaeozoic and Mesozoic bedrock stratigraphy for ~40 km to the east of the Thakkhola graben^{23–25}; we will refer to these structures, together with the synpositional faults found in the graben, as the Thakkhola fault system. Faults of this system are confined to the region north of the high peaks of the Himalaya, structurally above the lowermost shear zones of the South Tibetan detachment system^{25,26}.

Our study has focused on some of the easternmost structures of the Thakkhola fault system in the northern part of the Marsyandi river valley (Fig. 2). The most significant of these are normal faults developed in essentially unmetamorphosed Palaeozoic and Mesozoic rocks. They strike N20°E–N40°E and dip 45–60° northwest, similar in orientation to bounding faults of the Thakkhola graben, although we consider them to be older and related to the earliest stages of east–west extension. Striae on exposed fault surfaces are oriented N90°E ± 10°. Kinematic indicators, including slickenside surface characteristics and orientations of extensional fractures within discrete fault zones, demonstrate that displacement was primarily hanging wall down to the WNW. These oldest and easternmost faults of the Thakkhola system cut mylonitic fabrics related to structurally high shear zones of the South Tibetan detachment system, and are

FIG. 1 Generalized tectonic map of the Tibetan plateau (adapted from refs 9 and 11) showing the location of major structural features discussed in the text, and the area of Fig. 2.

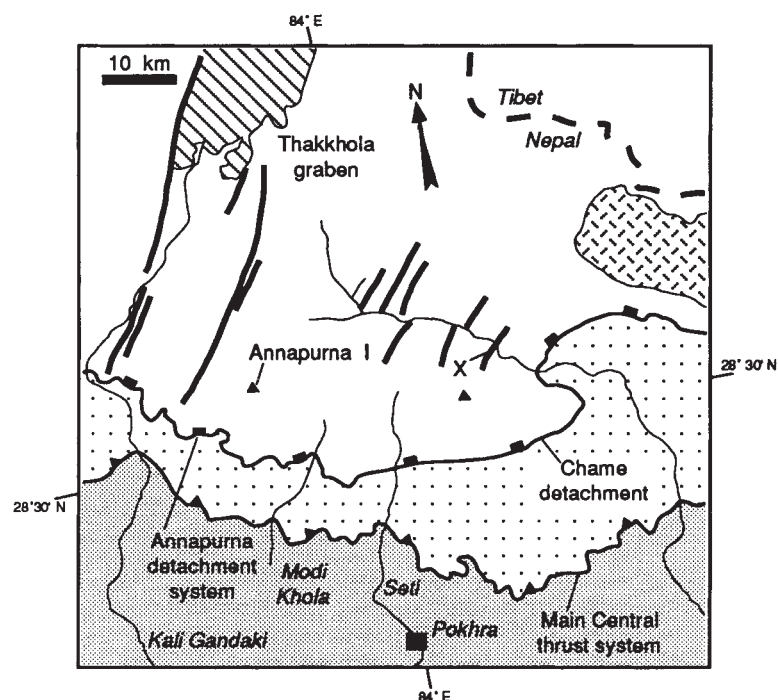
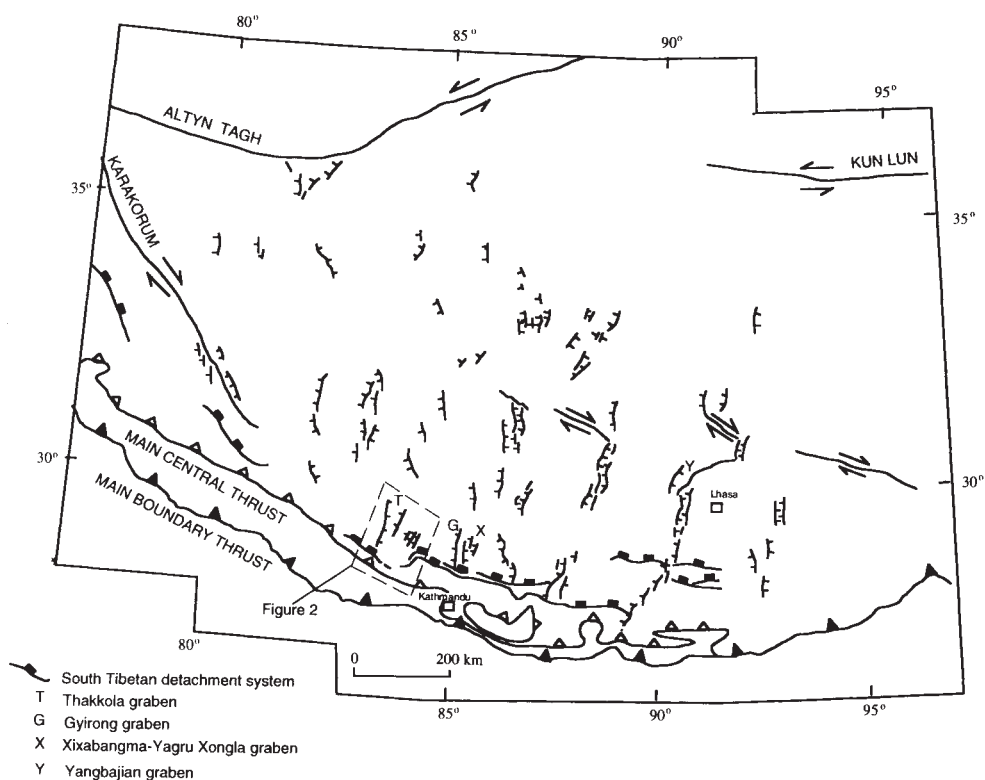


FIG. 2 Simplified geological map of the Annapurna region. X marks the sample location. €, Cambrian; K, Cretaceous; p€, Precambrian; Mz, Mesozoic; Pz, Palaeozoic; Plio, Pliocene.

TABLE 1 $^{40}\text{Ar}/^{39}\text{Ar}$ data for hydrothermal muscovite, Thakkola fault system

Tube current (A)	$^{36}\text{Ar}/^{40}\text{Ar}$	$^{39}\text{Ar}/^{40}\text{Ar}$	$^{39}\text{Ar}_K$ ($\times 10^{-14}$ mol)	Cum. $^{39}\text{Ar}_K$ (%)	$^{40}\text{Ar}^*$ (%)	Age (Myr)
Split no. 1						
11.5	0.00322	0.00694	0.004	0.12	4.71	7.2 ± 0.3
12.5	0.00228	0.02939	0.007	0.34	32.74	11.9 ± 0.5
13	0.00256	0.02851	0.014	0.77	24.33	9.1 ± 0.3
13.5	0.00122	0.05077	0.095	3.66	63.96	13.4 ± 0.5
14	0.00081	0.05709	0.192	9.51	76.03	14.2 ± 0.5
14.5	0.00033	0.06555	0.311	19.01	90.16	14.7 ± 0.6
15	0.00033	0.06895	0.208	25.36	90.40	14.0 ± 0.5
16	0.00049	0.06333	0.332	35.48	85.54	14.4 ± 0.5
18	0.00043	0.06303	0.826	60.67	87.26	14.8 ± 0.6
20	0.00033	0.06865	1.289	100.00	90.17	14.0 ± 0.5
					Average	14.3 ± 0.1
Split no. 2						
14	0.00100	0.05075	0.168	6.49	70.57	14.8 ± 0.6
16	0.00042	0.06430	0.904	41.43	87.62	14.5 ± 0.6
18	0.00035	0.06596	1.304	91.79	89.77	14.5 ± 0.6
20	0.00022	0.06905	0.149	97.53	93.39	14.4 ± 0.5
22	0.00035	0.07043	0.064	100.00	89.78	13.6 ± 0.5
					Average	14.5 ± 0.3

$^{39}\text{Ar}_K$, the number of moles of K-derived ^{39}Ar released during each heating step, and Cum. $^{39}\text{Ar}_K$, the cumulative percentage after each heating increment; $^{40}\text{Ar}^*$ (%), the percentage of radiogenic ^{40}Ar in the total ^{40}Ar for each analysis. Average ages are means weighted by the amount of $^{39}\text{Ar}_K$ in each increment, with uncertainties corresponding to 2 standard errors of the mean.

thus demonstrably younger²⁵. The age of the mylonitic fabrics associated with the South Tibetan detachment system in central Nepal is constrained by cross-cutting leucogranites (such as the Manaslu pluton) to be at least 22 Myr old^{27,30}.

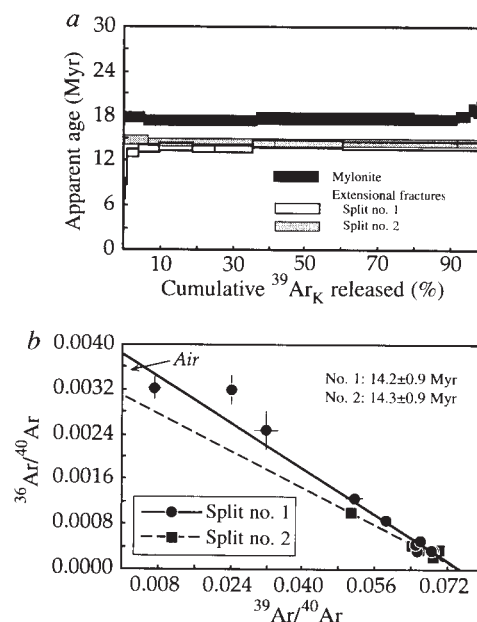
The Thakkhola fault system also includes vertical extensional fractures, striking $\text{N}0^\circ\text{E} \pm 10^\circ$, which are pervasive in Palaeozoic rocks of the upper Marsyandi valley. Many of these fractures contain minerals precipitated from late-stage hydrothermal fluids, apparently injected during extensional faulting. One of the more common vein materials is muscovite, which provides the opportunity to place minimum age constraints on extensional fracturing through $^{40}\text{Ar}/^{39}\text{Ar}$ dating. We applied this technique to coarse-grained muscovite ($\text{Si}^{\text{IV}} = 3.20$ per formula unit, $(\text{Fe}_{\text{total}} + \text{Mg})/\text{Al}^{\text{VI}} = 0.145$, where superscript IV or VI refers to a crystallographic site with tetrahedral or octahedral coordination, respectively) from a fracture developed in a lower amphibolite facies, impure metacarbonate rock from the Annapurna Yellow Formation³¹. The rock itself contained disseminated, fine-grained muscovite that grew before extensional fracturing.

To ensure that our sample of hydrothermal muscovite was not contaminated by older muscovite, the rock was broken along the fracture and 10–15 single-crystal fragments of hydrothermal muscovite were hand-picked from the cleavage surface under a binocular microscope. The material was then split in two fractions that were analysed separately to test for reproducibility of the results.

The two splits exhibited easily interpretable behaviour during laser incremental heating (Table 1; Fig. 3). The first was analysed in the most detail, yielding a relatively flat age spectrum with an average age of 14.3 ± 0.1 Myr. Split no. 2 had a statistically indistinguishable average age of 14.5 ± 0.3 Myr. Data from both splits defined linear arrays on an inverse isotope correlation diagram³², indicating isochron ages of 14.2 ± 0.9 Myr (split no. 1) and 14.3 ± 0.9 Myr (split no. 2). Increments from split no. 1 show greater dispersion on the correlation diagram and provide a better constraint on the initial $^{40}\text{Ar}/^{36}\text{Ar}$ ratio of these micas, but both regression curves indicate an initial ratio within uncertainty of the present-day atmospheric value.

FIG. 3 Incremental release spectrum (a) and inverse isotope correlation (b) diagrams for hydrothermal muscovites from the Thakkola fault system and metamorphic muscovite from the surrounding mylonite (errors at 2σ). ('Cumulative Ar_K ' is defined in Table 1.) Uncertainties for samples shown without error bars in b are smaller than the designating symbols. Solid and dashed lines in b designate least-squares linear regression fits using the algorithm of York³⁷.

METHODS. Samples were wrapped in cadmium foil and irradiated before analysis in the McMaster University research reactor in Hamilton, Ontario, Canada. Salts included in the irradiation package were used to correct for interference by reactor-induced masses. The fast-neutron fluence during irradiation was monitored using Fish Canyon sanidine (27.84 Myr); ages and age uncertainties were calculated using an estimated irradiation parameter (J) of 0.00059 ± 0.00001 (2σ). Incremental heating experiments were done at the Massachusetts Institute of Technology using a 10-W argon-ion laser with the technique described by Hodges³⁸.



To evaluate the possibility that, despite our best efforts, the analysed micas were somehow relicts from the earlier mylonitic fabric rather than neoblasts crystallized during hydrothermal activity, we also dated compositionally similar muscovite ($\text{Si}^{\text{IV}} = 3.24$ per formula unit; $(\text{Fe}_{\text{total}} + \text{Mg})/\text{Al}^{\text{VI}} = 0.141$) that defines the mylonitic foliation within the South Tibetan detachment zone in this area. Data for this mica defined a plateau age of 17.6 ± 0.3 Myr (Fig. 3), an inverse isochron age of 17.4 ± 0.7 Myr, and an initial ratio within uncertainty of the present-day atmosphere. The ages of the micas in the mylonite and the micas in the extensional fractures are significantly different (at a confidence level of $>95\%$), demonstrating that the micas separated from the fracture grew after cooling of the surrounding mylonitic rocks to temperatures below ~ 625 K (the nominal closure temperature for Ar diffusion in muscovite³³) and that the ~ 14 -Myr age does not reflect the long-term thermal effects of pre-22 Myr igneous activity.

We interpret the hydrothermal muscovite data as strong evidence that east-west extension began before 14 Myr ago in the area corresponding to the present-day southern margin of the Tibetan plateau. This finding supports earlier suggestions of an age of at least 13 Myr for the plateau based on the $^{40}\text{Ar}/^{39}\text{Ar}$ age of volcanic rocks that are thought to have been derived from thinned mantle lithosphere beneath Tibet³⁴. It is also consistent with interpretations relating geochronological evidence for rapid cooling of rocks in southern Tibet to Early Miocene plateau uplift^{10,35}.

Previous studies in southern Tibet suggested that east-west extension had not progressed to the point that sedimentary basins, such as the Thakkola graben, began to form until the Pliocene epoch; however, the existence of significantly older, north-striking normal fault systems implies that the stress field responsible for east-west extension had developed by the Middle Miocene epoch. If the onset of east-west extension marks the beginning of accelerated uplift of the plateau due to an increase in the potential energy of the Tibetan lithosphere⁹, then our data imply that uplift was not simultaneous with the marked change in South Asian climate at ~ 8 Myr ago but began several million years earlier. Future models of Cenozoic global climate change must be reconciled with a growing body of geological evidence suggesting that the Tibetan plateau was an established geomorphologic feature at least as early as Middle Miocene time, possibly as old as Palaeogene³⁶. □

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Ocean-ridge basalts with convergent-margin geochemical affinities from the Chile Ridge

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COLLISIONS between active spreading centres and subduction zones have occurred frequently throughout Earth history¹. Of the few sites of ridge subduction active today, the southern Chile Ridge has the simplest tectonic history². We report here that basalts recovered from the Chile Ridge adjacent to the Chile Trench have geochemical characteristics unlike those of mid-ocean-ridge basalts sampled elsewhere, and show affinities with arc volcanics. The observed chemical variations are consistent with contamination of depleted sub-oceanic mantle by marine sediments and altered oceanic crust, which may have been derived from the adjacent subduction zone. Although some ocean islands show evidence of crustal recycling^{3–5}, the Chile Ridge lavas are the first ocean-ridge basalts to exhibit such extreme geochemical characteristics, and may thus offer the opportunity to study the development of one type of mantle heterogeneity as it occurs. The discovery of ocean-ridge basalts with convergent-margin chemical characteristics suggests that caution is warranted in using trace-element systematics to identify the provenance (mid-ocean ridge or supra-subduction zone) of ophiolites^{6,7}.

The Chile Ridge (60 mm yr⁻¹ total spreading rate), which separates the Nazca and Antarctic plates, is currently being subducted beneath the westward-migrating South American plate (Fig. 1)². In January 1993, we sampled 440 km of ridge axis along the four ridge segments nearest the trench (66 stations). All analyses reported here (direct-current plasma emission spectrometry, inductively coupled plasma mass spectrometry, thermal ionization mass spectrometry) were performed on fresh, hand-picked glasses; representative analyses are presented in Table 1. Major-element data⁸ show that the ridge axis samples are of mid-ocean-ridge basalt (MORB) composition, represent diverse parental magma compositions, and exhibit a range in extent of fractionation ($\text{Mg}\# = 0.42\text{--}0.65$), although most are relatively undifferentiated ($\text{Mg}\# > 0.6$). ($\text{Mg}\#$ is the Mg number, defined as $100\text{Mg}/(\text{Mg} + \text{Fe})$.) Here, we focus on the unusual trace-element systematics of these lavas; detailed descriptions of the major-element and isotope variations will be presented elsewhere.

TABLE 1 Representative analyses of Chile Ridge glasses

	Segment 1		Segment 2	Segment 3		Segment 4	
	D14-9	D20-1	D34-1	D42-4	D53-2	D63-5	D61-1
Latitude (°S)	45.953	46.041	45.781	45.642	44.539	43.873	44.464
Longitude (°W)	75.934	75.897	76.780	77.889	78.315	82.427	82.180
Depth (m)	3,280	3,296	4,010	2,918	3,490	3,895	4,180
SiO ₂	50.46	50.53	50.02	50.58	50.06	48.23	50.09
Al ₂ O ₃	15.17	15.44	15.10	15.95	15.71	16.95	14.88
Fe ₂ O ₃	9.82	9.68	10.34	9.16	9.56	10.60	10.67
MgO	8.56	7.97	8.63	8.18	8.68	9.23	8.28
CaO	12.40	11.57	11.34	11.65	11.83	10.68	11.04
Na ₂ O	2.69	2.85	2.76	2.78	2.36	2.80	2.69
K ₂ O	0.070	0.169	0.046	0.446	0.089	0.450	0.075
TiO ₂	1.22	1.30	1.35	1.18	1.12	0.95	1.43
MnO	0.167	0.162	0.171	0.154	0.159	0.172	0.176
P ₂ O ₅	0.124	0.134	0.116	0.169	0.129	0.168	0.147
Sum	100.67	99.80	99.87	100.26	99.69	100.22	99.47
Ba	14.4	27.8	13.1	163	14.5	115	17.4
Sr	123	131	88	255	103	150	100
Zr	87	99	89	100	91	93	99
Y	29.0	29.3	32.5	23.9	29.5	25.3	36.3
V	267	265	282	253	255	207	306
Sc	38.5	37.4	36.9	35.6	35.9	35.0	37.1
Cu	78	66	66	84	71	75	66
Cr	377	320	317	305	351	307	290
Ni	95	98	136	95	133	164	133
Pb	0.332	0.768	0.315	1.313	0.348	1.054	0.322
Th	0.132	0.486	0.117	1.441	0.175	1.132	0.166
U	0.033	0.131	0.023	0.379	0.049	0.245	0.061
Rb	1.02	4.44	1.11	12.3	1.35	11.4	1.31
Cs	0.015	0.171	0.014	0.362	0.031	0.143	0.013
Nb	1.76	2.30	1.20	5.81	1.75	10.2	2.86
Hf	1.99	2.38	2.28	2.21	2.04	1.93	2.73
Ta	0.119	0.166	0.078	0.347	0.116	0.595	0.191
La	2.52	3.55	1.89	7.88	2.66	8.41	3.39
Ce	7.91	10.45	7.18	18.88	8.29	16.48	10.80
Pr	1.46	1.75	1.45	2.71	1.49	2.06	1.88
Nd	8.02	9.26	8.57	12.56	8.00	8.71	10.26
Sm	2.81	3.06	3.15	3.35	2.74	2.35	3.67
Eu	1.04	1.10	1.12	1.17	0.94	0.87	1.26
Gd	3.85	4.27	4.44	3.81	3.81	3.13	5.16
Tb	0.740	0.808	0.852	0.682	0.737	0.618	0.984
Dy	4.59	4.95	5.30	3.95	4.61	3.93	6.40
Ho	1.00	1.08	1.16	0.83	1.02	0.90	1.42
Er	2.71	2.97	3.18	2.23	2.84	2.52	3.90
Tm	0.424	0.468	0.497	0.335	0.439	0.415	0.601
Yb	2.71	2.90	3.12	2.14	2.83	2.71	3.76
Lu	0.416	0.450	0.485	0.332	0.443	0.430	0.583
²⁰⁶ Pb/ ²⁰⁴ Pb	18.165	18.398	17.930	19.489	18.307	17.953	18.133
²⁰⁷ Pb/ ²⁰⁴ Pb	15.519	15.553	15.490	15.688	15.541	15.524	15.496
²⁰⁸ Pb/ ²⁰⁴ Pb	37.912	38.179	37.689	39.556	38.032	38.314	37.713
⁸⁷ Sr/ ⁸⁶ Sr	0.702668	0.702719	0.702674	0.704072	0.702487	0.703552	0.702554
¹⁴³ Nd/ ¹⁴⁴ Nd	0.513149	0.513113	0.513162	0.512709	0.513146	0.512900	0.513188

Sampling locations (segments 1–4) are shown in Fig. 1. Major-element (wt% oxide) and trace-element (p.p.m.; Ba, Sr, Zr, Y, V, Sc, Cu, Cr, Ni) analyses were performed by direct-current plasma emission spectrometry at Duke University³²; U, Th, Pb, Cs, Rb, Nb, Hf, Ta and rare-earth-element (p.p.m.) analyses were performed by inductively coupled mass spectrometry at Cornell University in the laboratory of W. White³³, following sample preparation at Duke University; reproducibility for inductively coupled plasma mass spectrometry analyses, as judged by replicate dissolutions, are ± 1 –3% for rare-earth elements and ± 1 –6% for other elements. Nd, Sr and Pb isotope analyses were performed at the University of North Carolina, Chapel Hill (sources for analytical techniques summarized in ref. 34). Isotope data are normalized values of 0.11940 for ⁸⁶Sr/⁸⁸Sr, and 0.72190 for ¹⁴⁶Nd/¹⁴⁴Nd. Measured replicate standard values are 0.710243 for ⁸⁷Sr/⁸⁶Sr for NBS-987, and 0.511855 and 0.512136 for ¹⁴³Nd/¹⁴⁴Nd for the La Jolla and Ames standards, respectively. Pb data are referenced to the following values for NBS-981: 16.937, 15.491 and 36.721 for ²⁰⁶Pb/²⁰⁴Pb, ²⁰⁷Pb/²⁰⁴Pb and ²⁰⁸Pb/²⁰⁴Pb, respectively (replicate standard analyses yield a mean correction factor of 0.13% per atomic mass unit (AMU)). Duplicate Pb isotope measurements agree to better than 0.05% per AMU. Measured analytical uncertainties ($2\sigma/\sqrt{N}$) for both Sr and Nd isotope ratios $< \pm 0.000015$. Typical blank values for Sr, Nd and Pb average 0.3, 0.2 and 0.4 ng, respectively.

Trace-element ratios used to distinguish MORB and ocean island basalts (OIB) from arc lavas and continental materials (Fig. 2) clearly show that many southern Chile Ridge lavas are unlike the majority of MORB and OIB analysed to date⁵, including MORB recovered >600 km farther northwest on the Chile Ridge (L. Dosso, personal communication). On these plots, samples from segment 1 (closest to the trench) and segment 3 form subparallel trends extending well outside the MORB/OIB fields to low Nb/U, Ce/Pb and Rb/Cs and high Th/La, towards values more commonly associated with arc volcanics or continental crust^{9–11}. Segment 4 basalts also trend toward low Ce/Pb and high Th/La, but at relatively high, MORB-like values of Nb/U. Trace-element abundance patterns normalized to the composition of normal MORB (N-MORB) (Fig. 3) show that the most enriched samples from segments 1 and 3 are also similar

to arc lavas in their highly elevated abundances of the most incompatible trace elements, enrichment in Pb (relative to Ce and Sr), and depletion in Nb and Ta (relative to U and K). But in spite of the relative depletion in Nb and Ta in these enriched samples, the absolute abundances of these elements are equal to or greater than those of N-MORB. In contrast, enriched samples from segment 4 display little or no relative depletion in Nb and Ta and have enrichments more typical of OIB. Interestingly, although segments 1 and 3 display similar and unusual behaviour in the trace-element ratios shown in Fig. 2, they differ dramatically in their rare-earth element patterns: segment 3 includes samples both enriched and depleted in light-rare-earth elements (chondrite-normalized La/Sm = 0.6–1.5), whereas segment 1 samples are all light-rare-earth-depleted ((La/Sm)_N < 0.7; see Table 1 for absolute abundances).

The anomalous trace-element ratios shown in Fig. 2 are likely to represent anomalous ratios in the mantle sources, as the elements in each ratio (except Th and La) are not believed to fractionate from one another during melting in the sub-oceanic mantle or during crystallization⁵. The observation that samples from segments 1 and 3 extend from N-MORB compositions towards these anomalous compositions suggests that the depleted N-MORB source in this region has been variably contaminated by other materials. For segment 4 basalts, the contaminant has isotopic characteristics (Table 1) which are most similar to those associated with the Indian Ocean geochemical province^{12,13}. The Indian Ocean province is related to the globe-encircling belt of anomalous isotopic compositions known as the Dupal anomaly¹⁴ centred at 30° S, and the segment 4 lavas may represent the first evidence of the Dupal anomaly in Pacific MORB¹⁵. The geochemical systematics of segment 4 lavas will be explored in future work; here we focus on the systematics of segments 1 and 3, because they display the most dramatic departures from MORB compositions analysed to date.

The first question to be addressed is the nature of the contaminant(s) for segments 1 and 3. The affinity of some Chile Ridge lavas with arc or back-arc compositions suggests that they may have a similar origin. It is now generally accepted that many arc lavas have trace-element and isotopic signatures influenced by melts or fluids derived from marine sediments and altered oceanic crust associated with the subducting slab (for example, refs 16–18) and the trace-element trends observed for segments 1 and 3 (Fig. 2) are qualitatively consistent with contamination by such materials. To evaluate quantitatively the feasibility of contamination by sediment and altered crust, we have investigated a simple contamination model. In this model, slab-derived fragments of sediment and altered crust are mixed convectively with sub-ridge mantle to form localized heterogeneities; during upwelling, these heterogeneities melt and these contaminated melts mix with typical MORB melt produced from depleted mantle in the same melting regime. Calculated mixing trends between MORB melts and the endmember contaminated melts have been compared to the trace-element data of Fig. 2a, b and presented in Fig. 4 (see Fig. 4 legend for calculation details).

Contamination by marine sediment alone (model A) does not reproduce the trends observed along segments 1 and 3 (Fig. 4).

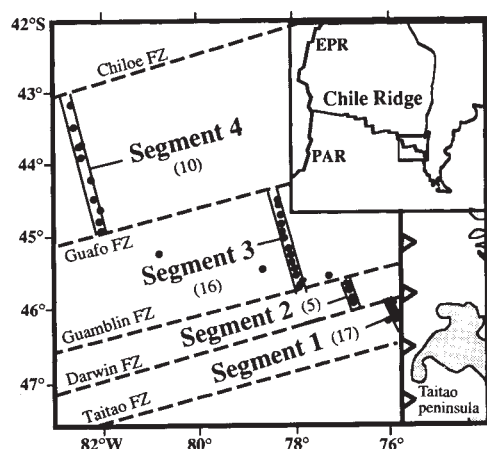


FIG. 1 Tectonic elements of the southern Chile Ridge in the vicinity of its intersection with the Chile Trench². Ridge segments (double lines) with dredging and wax coring sampling sites (filled circles) are numbered 1–4 with increasing distance from the trench (barbed vertical line); the number of sampling sites along each ridge segment is indicated in parentheses; fracture zones (FZ) are shown as dashed lines. Also shown is the location of the Taitao peninsula which hosts the Taitao ophiolite and extends offshore as the submarine Taitao ridge^{30,31}. Inset, regional location of the study area (box): EPR, East Pacific Rise; PAR, Pacific–Antarctic ridge.

This finding, which is robust over a range of common marine sediment compositions¹⁷, also argues against contamination by sediment incorporation during eruption of the magmas, which could have occurred along the heavily sedimented segment 1 ridge axis. Furthermore, the fact that segment 3 shows a smooth gradient in composition over its entire 150 km length, with decreasing enrichment from south to north, argues against shal-

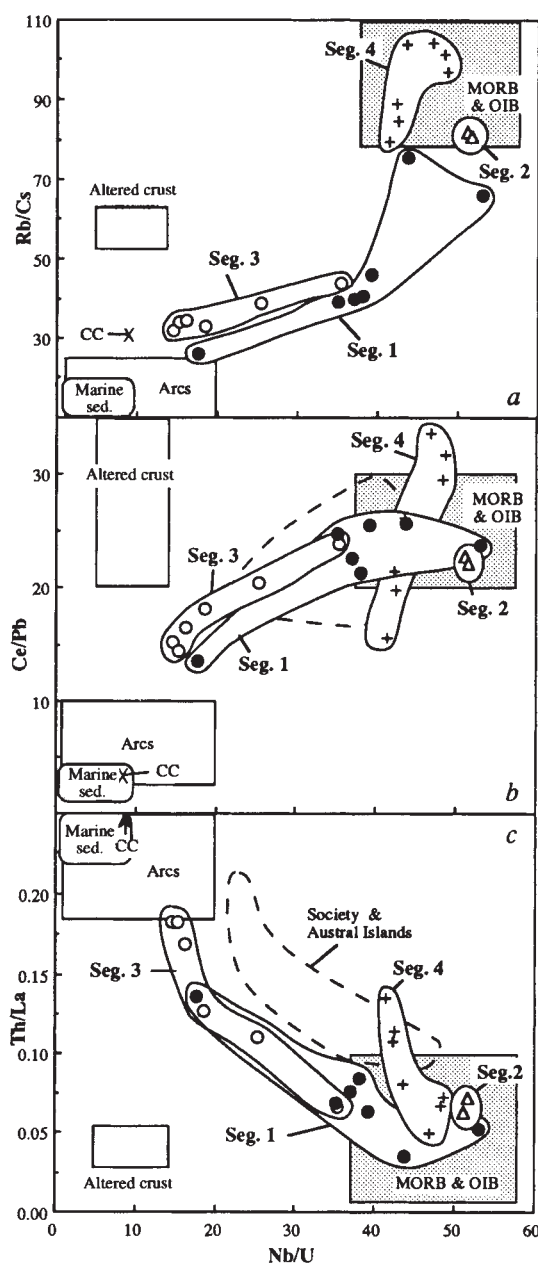
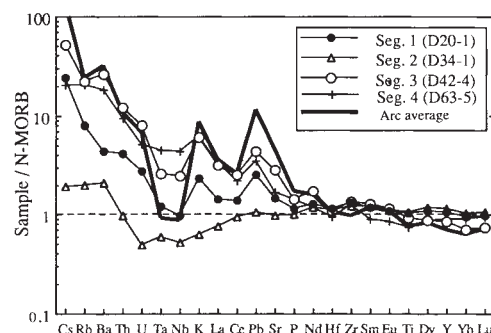


FIG. 2 The Nb/U ratio plotted against Rb/Cs (a), Ce/Pb (b) and Th/La (c) for Chile Ridge glasses. Segment 1 (filled circles); segment 2: (open triangles); segment 3 (open circles); segment 4 (crosses). Also shown are fields for MORB and ocean islands^{5,9,10}, marine sediments^{17,18,35}, arc lavas¹⁰, altered oceanic crust (estimate modified from refs 19, 28, 36, 37) and average upper continental crust (cross labelled 'CC')¹¹. Data from the Society and Austral islands⁴, which exhibit trace-element and isotopic evidence of sediment and altered crust recycling^{3,4,28}, are shown as fields bounded by dashed lines extending outside the MORB/OIB fields on panels b and c. (The two samples from segment 1 with the highest Rb/Cs ratio have Cs abundances of 12–15 p.p.b., and inaccuracies in measurement at these low abundances may contribute to their departure from the trend. For a similar reason, two samples with <6 p.p.b. Cs from segments 2 and 4 have been omitted from this figure.)

FIG. 3 Trace-element abundance patterns for representative Chile Ridge glasses (symbols as in Fig. 2) and an average arc lava normalized to the composition of N-MORB⁵. Only enriched samples from segments 1, 3 and 4 are shown; depleted samples from these three ridge segments (see Table 1) display patterns and abundances similar to that shown for the segment 2 sample. Average arc-lava composition is based on the data of refs 10, 38 and T. Plank, personal communication; Ta abundances have been calculated from Nb data, assuming $Ta = Nb/17$.



low crustal assimilation processes as the source of contamination.

Modelling with contaminants that include mixtures of both sediment and altered crust generally produce the same trends as the segment 1 and 3 trace-element data. Two of these model calculations, using different proportions of sediment and altered crust, are shown in Fig. 4a, b (models B and C). The relatively low Rb abundances (<5 p.p.m.) of segment 1 lavas, combined with their trend toward low Rb/Cs (to 26) can be modelled by mixing of MORB melt with melts of mantle containing 0.4% sediment and 1.6% altered oceanic crust (model B). To produce the high incompatible-trace-element abundances of segment 3 (for example, Rb up to 14 p.p.m.) and higher Rb/Cs ratios (~35), a greater total amount of contaminant with a greater proportion of altered crust is suggested (~0.5% sediment and ~7.5% altered crust; model C). High U/Pb ratios in samples from segment 3 (to 0.3) relative to unaltered MORB (~0.15)⁵ are also consistent with incorporation of altered crust (with $U/Pb > 0.45$)¹⁹ and cannot be explained by contamination solely by marine sediments which are generally characterized by low U/Pb ratios (<0.1)¹⁷. It is important to note that La/Nb, which is particularly high in arc lavas, is not appreciably affected by mixing with the small amount of sediment suggested; thus the MORB-like La/Nb ratios (~1.5)⁵ of the Chile Ridge lavas are also consistent with the modelling. Although these simple calculations do not provide a unique solution, they nevertheless place some constraints on the amounts of contaminants, and suggest that, as in arc lavas, the distinctive trace-element signatures of segments 1 and 3 may have been produced by contamination with varying proportions of sediment and altered crust or melts/fluids derived therefrom.

How and when were these contaminants introduced, and is there a relationship with the peculiar tectonic setting? The geographical proximity of the Chile Ridge to the Chile Trench raises the possibility that the sub-oceanic mantle in this region has become contaminated with material derived directly from the adjacent subduction zone, either from the slab itself or from the slab-contaminated sub-arc mantle. It is interesting to note that arc-affinity lavas have also been recovered in advance of the subduction zone in the Woodlark basin, the other site of active ridge subduction, although the tectonic history of the region has been more complex²⁰.

At present, we can only speculate on the mechanisms and pathways for entraining the appropriate materials from the subduction zone and circulating them back beneath the ridge crest, but several different processes are possible. When a ridge approaches a trench, the slab entering the subduction zone is young, hot and buoyant. Resistance to subduction may cause breakup of the brittle portions of the slab²¹. Absence of seismicity deeper than the 100 km near the Chile triple junction suggests that the warm plate is rapidly reheated to upper-mantle temperatures, and this may result in shallow resorption of the plate materials²². Thus fragments of the slab may either founder or be sheared into the adjacent sub-oceanic mantle, be carried westward by the shallow asthenospheric flow postulated for this

region²³, and entrained in the upwelling and melting regime beneath the adjacent ridge. Alternatively, the previously active ridge segment which was subducted just south of this region may have undergone continued extension along the plate boundary without crustal accretion, resulting in a 'slab window'²⁴. This gap in the plate could provide an avenue of communication between the contaminated sub-arc mantle and the adjacent sub-oceanic mantle. Regardless of the specific mechanism, it is clear from the geochemical diversity of the region that the contaminants are irregularly dispersed in the sub-ridge mantle. Future analyses of fluid-mobile elements (such as boron)²⁵ and their relationship to fluid-immobile elements (such as thorium) will be used to distinguish between direct mixing with slab fragments and mixing with sub-arc mantle that was previously metasomatized with slab-derived fluids.

The isotope data for these samples provide an additional test of the feasibility of contamination from the adjacent subduction zone. Considering first segment 1, and using isotope ratios for Nazca plate sediments recovered >1,000 km from our study area^{26,27}, addition of 0.4% sediment and 1.6% altered crust (as calculated from trace elements for segment 1 above) to a MORB source leads to only slight increases in each of the Pb isotope ratios, and minor decreases in $^{143}Nd/^{144}Nd$, as is observed (Table 1); these slight Pb changes correlate with the trace-element indices of contamination (Fig. 4c). Calculated Sr isotope compositions for the most enriched segment 1 samples (using 0.708, 0.7045 and 0.7025 for sediment, altered crust and MORB melt/mantle) results in somewhat higher Sr isotope ratios (0.7028) than observed (~0.7027), but given the uncertainties regarding sediment and altered crust compositions, this discrepancy is small. The isotopic variations observed along segment 1 are thus consistent with recent contamination by sediment and altered oceanic crust (Fig. 4c) introduced from the nearby subduction zone.

Explaining the segment 3 lava compositions by recent contamination at the adjacent subduction zone is more problematical, however, because an endmember with the extreme Pb isotope ratios of segment 3 lavas (for example, $^{206}Pb/^{204}Pb$ up to 19.5; Table 1) has not been documented in Nazca plate sediments to the north^{26,27}, and most of the likely South American sources for terrigenous sediment input are relatively young and of intermediate Pb isotopic composition¹⁰, although some recently analysed samples from the nearby Meseta Central have $^{206}Pb/^{204}Pb$ up to 19.4 (M. L. Goring, personal communication). Furthermore, the metasedimentary rocks that form the basement of the arc and accretionary wedge east of the triple junction have Nd model ages that suggest a Proterozoic sedimentary precursor (J. Tarney, personal communication), and it is possible that the recycled component for segment 3 may include such material eroded from the continental margin. Alternatively, the contaminant for segment 3 may have been introduced into the mantle during an ancient subduction and deep recycling event, as has been suggested for the Society Islands^{4,28} (which are distinct from other ocean islands; Fig. 2), in which case it would have nothing to do with the modern tectonic setting. It is important

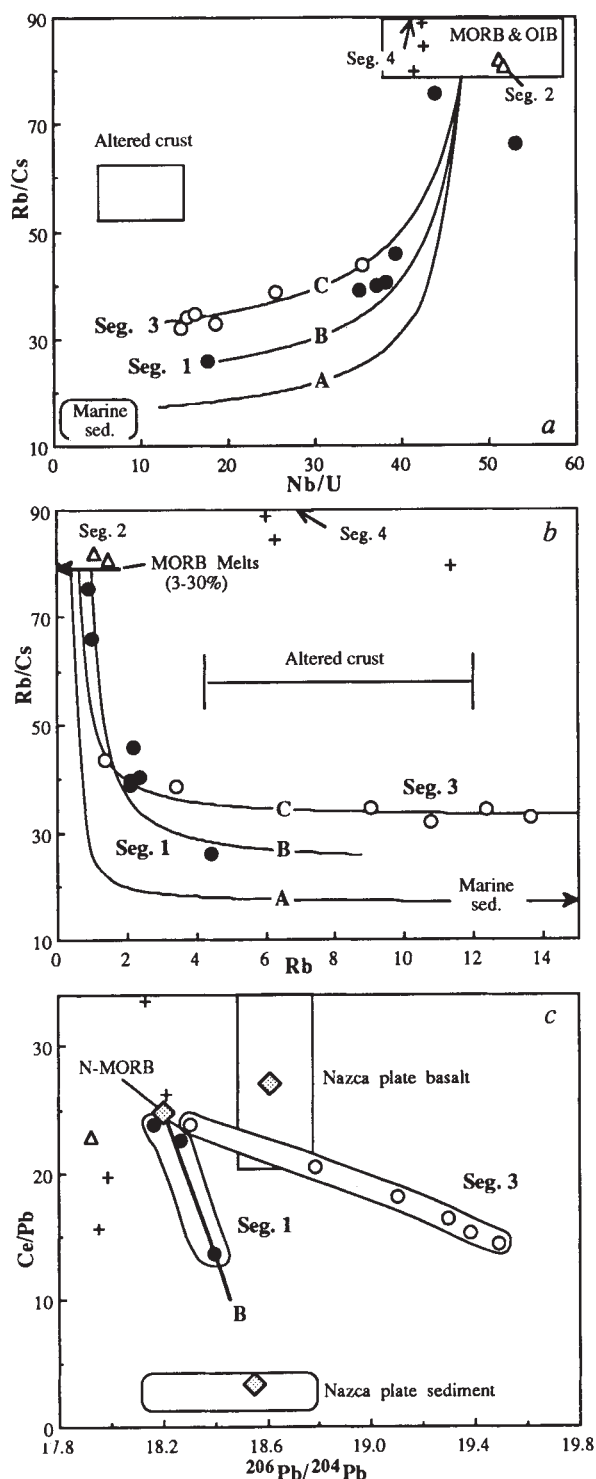


FIG. 4 Trace-element and isotopic variations in Chile Ridge glasses compared to model calculations (symbols and fields as in Fig. 2). a, Nb/U versus Rb/Cs; b, Rb (p.p.m.) versus Rb/Cs; c, $^{206}\text{Pb}/^{204}\text{Pb}$ versus Ce/Pb. In b, Rb concentrations of segment 1 samples with Rb/Cs of 38–47 have been corrected to 8 wt% MgO to minimize fractionation effects; all other samples have MgO $\geq$ 8 wt%. Model calculations involve mixing sediment, altered crust and mantle in varying proportions. This contaminated mantle is then melted to 5% batch melting; the calculated trends (models A, B and C) show mixing between contaminated melt and normal MORB melts produced by varying extends of melting. Model A, mixing between MORB melt (15% melting) and melt produced from mantle contaminated with 2% sediment; model B, mixing between MORB melt (6% melting) and mantle contaminated with 0.4% sediment and 1.6% altered crust; model C, mixing between MORB melt (10% melting) and mantle contaminated with 0.48% sediment and 7.52% altered crust. For these calculations, Rb, Cs, Nb, U, Ce and Pb in sediment^{17,18,35,36} are respectively 60, 3.6, 10, 1.6, 73 and 20 p.p.m.; in altered crust are 9.6, 0.153, 1.22, 0.275, 6.19 and 0.23 p.p.m. (ref. 37 and T. Plank, personal communication; Pb estimated from ref. 28); and in mantle source are 0.063, 0.0008, 0.249, 0.0053, 0.775 and 0.031 p.p.m. (estimate derived from refs 5, 36, 39 and Ce/Pb = 25, Nb/U = 47). Bulk distribution coefficients³⁸ for these elements for melting calculations are respectively 0.002, 0.002, 0.008, 0.008, 0.015 and 0.015. Note that a marine sediment of intermediate composition compared to the global range^{17,18,35} was used in these calculations because full trace-element analyses are not available for the sediment section being subducted in this region. In c, fields show ranges of $^{206}\text{Pb}/^{204}\text{Pb}$ for Nazca plate sediments and basalt; average compositions (large stippled diamonds) for modelling are 18.55 and 18.6 for respectively Nazca plate sediment and basalt^{26,27}, and 18.2 for MORB and mantle source; average Ce/Pb taken from references above.

venance of ophiolitic rocks. It has been proposed that processes associated with ridge subduction may be linked with ophiolite obduction; for example, slab breakup and subsequent underthrusting during continued subduction may cause obduction of slab pieces into the forearc region²¹. If our segment 1 or 3 lavas were to comprise this obducted slab fragment, the ophiolite would display trace-element systematics more characteristic of arc (or back-arc) lavas than of MORB (Fig. 3). Volcanic rocks from the Taitao ridge and Taitao ophiolite, located just south of the triple junction and emplaced $\sim$ 4 Myr ago in conjunction with previous subduction of the Chile Ridge, include basaltic rocks with enriched arc-like or continental chemical signatures^{30,31}. For ophiolites where the tectonic setting of emplacement is less clear, the use of diagnostic trace-element signatures (for example, refs 6, 7) to discriminate petrogenetic provenance could lead to the erroneous conclusion that the ophiolite accreted in a supra-subduction-zone environment, as is now assumed for most ophiolites⁷. In view of the findings from the Chile Ridge, where subduction zone signatures have been produced at a mid-ocean ridge, the use of such trace-element discriminants should be approached with caution. □

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to note, however, that although segment 3 lavas show isotopic affinities with San Felix and Juan Fernandez islands, located $>1,000$ km north of our study area²⁹, these ocean islands do not display the trace-element systematics indicative of crustal recycling as are found in segment 3 lavas (C. Devey, personal communication). Thus, a hotspot-like origin for segment 3 is also problematical. In summary, the constraints we have at present do not preclude recent contamination for segment 3, but suggest at least a more complex scenario than that for segment 1, and involving contaminants of different isotopic compositions.

One important implication of this work concerns the common practice of using trace-element signatures to establish the pro-

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Reconstructing the evolutionary history of the artiodactyl ribonuclease superfamily

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THE sequences of proteins from ancient organisms can be reconstructed from the sequences of their descendants by a procedure that assumes that the descendant proteins arose from the extinct ancestor by the smallest number of independent evolutionary events ('parsimony')^{1,2}. The reconstructed sequences can then be prepared in the laboratory and studied^{3,4}. Thirteen ancient ribonucleases (RNases) have been reconstructed as intermediates in the evolution of the RNase protein family in artiodactyls (the mammal order that includes pig, camel, deer, sheep and ox)⁵. The properties of the reconstructed proteins suggest that parsimony yields plausible ancient sequences. Going back in time, a significant change in behaviour, namely a fivefold increase in catalytic activity against double-stranded RNA, appears in the RNase reconstructed for the founding ancestor of the artiodactyl lineage, which lived about 40 million years ago⁶. This corresponds to the period when ruminant digestion arose in the artiodactyls, suggests that contemporary artiodactyl digestive RNases arose from a non-digestive ancestor, and illustrates how evolutionary reconstructions can help in the understanding of physiological function within a protein family^{7–9}.

The RNase A superfamily includes proteins that display many interesting but poorly understood biological activities, including immunosuppressivity¹⁰, cytostatic activity¹¹, antitumour activity¹², endothelial-cell-stimulatory activity¹³, and lectin-like behaviour¹⁴, many of which have arisen by gene duplication since the time that mammals diverged from reptiles some

300 Myr ago. The abundance of RNase sequences from contemporary artiodactyls allows the reconstruction of the sequences of RNases that were the evolutionary intermediates in the most recent 40 Myr of this evolution (Table 1)¹⁵. Genes encoding the reconstructed proteins were obtained in the laboratory by site-directed mutagenesis from a synthetic gene for RNase⁷. The genes were then expressed in *Escherichia coli* and the resulting 'ancient' proteins purified to homogeneity using methods reported elsewhere^{16,18}.

To assess whether reconstruction by parsimony analysis yields proteins plausible as evolutionary intermediates in the evolution of the RNase family, the catalytic activities, substrate specificities, and thermal stabilities of the reconstructed RNases were examined. Most of the reconstructed proteins behave as expected for putative ancestral ruminant digestive RNases. This is particularly apparent when examining their kinetic properties (Table 2). Modern digestive RNases are catalytically active against small RNA substrates and single-stranded RNA¹⁹, so presumably correctly reconstructed ancestral digestive RNases should retain these properties. Consistent with these expectations, the k_{cat}/K_m values for the putative ancestral RNases with UpA as substrate do not differ substantially from those of contemporary bovine digestive RNase (Table 2)²⁰. The standard deviation of k_{cat}/K_m with UpA (uridylyl 3'→5' adenosine) as substrate among the reconstructed ancestral enzymes, is only 25%. With poly(U) as substrate, the deviation is even smaller (18%). Thus, based simply on catalytic power, the sequences reconstructed by parsimony make plausible ancestral pancreatic RNases. Further, if this *in vitro* behaviour alone is accepted as a measure, at least some of the changes in the sequences of ruminant pancreatic RNases over the past 40 Myr appear to have been neutral.

Next, modern digestive enzymes generally are known to be stable to thermal denaturation. To learn whether the putative ancient RNase sequences behaved as digestive enzymes by this criterion, denaturation temperatures were measured (Table 3)²¹. Again, little change was observed in thermal stability back to ancestor **h**. The experimental melting temperatures for these ancient proteins differed with a standard deviation of 1.1 °C when compared with RNase A; typical experimental errors were ±0.5 °C. Therefore, by thermostability data as well as kinetic data, the reconstructions obtained by parsimony analysis are reasonable, at least back to ancestor **h**.

For the more ancient ancestors **i** and **j**, however, thermal stability decreases. The decrease is small, but lies outside experimental error. Of course, this decrease may reflect an incorrect reconstruction, but the change in thermal stability appears in the evolutionary tree at approximately the same time as another change in behaviour, the catalytic power of the reconstructed RNase for the hydrolysis of the duplex RNA, poly(A)·poly(U) (Table 2). Bovine digestive RNase A has only low catalytic activity against duplex RNA under physiological conditions; such activity is presumably not needed for a digestive enzyme. Reconstructed RNases dating back to about 40 Myr behave similarly. This changes markedly, however, in the reconstructed ancestor **h** and its immediate predecessors. With these reconstructed enzymes, catalytic activity against the double-stranded RNA substrate poly(A)·poly(U) is about five times higher than in the RNases that evolved from it (Table 2).

These changes in molecular behaviour correspond to a point in the divergent evolution of mammals where digestive physiology in ungulates also underwent substantial changes, ultimately yielding artiodactyls with 'true ruminant' foregut digestion. In true ruminants (including oxen, deer and giraffe), bacterial fermentation takes place in a stomach (the rumen) preceding the main digestive chambers. This physiology appears to have substantial adaptive value in many herbivorous environments; it may have convergently evolved in marsupial kangaroos, the colobine monkey primates, and more than once within the artiodactyl lineage itself²². Ruminants ferment cellulose with

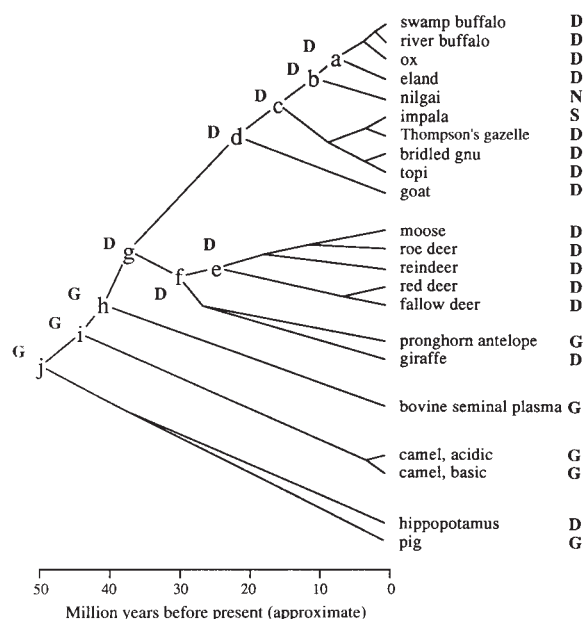


FIG. 1 The evolutionary tree used in this work. Lower-case letters in the nodes in the graph designate putative intermediates in the evolution of the protein family (Table 1). Upper-case letters (D and G) indicate the residue at position 38 in the contemporary and reconstructed RNases. The timescale is approximate. The tree was adapted from ref. 15 with a single alteration. In our tree, the pig and the hippopotamus are joined together in a separate subfamily that branches together from the main line of descent. In the Beintema-Fitch tree, the pig and the hippopotamus diverge from the main line at separate points. Our tree reflects the classical grouping of pig and hippopotamus into a suborder *Suina*, and therefore incorporates some biological information in addition to the RNases themselves. This change has two effects. It reduces the number of putative ancestors on the main line of divergence by one, and helps resolve several ambiguities in the Beintema-Fitch reconstructions (Table 1). The revision does not alter any unambiguously assigned amino acid in the Beintema-Fitch reconstructions. Details of the reconstructions are available (email sab@ezrl.vmsmail.ethz.ch). The placement of seminal RNase in the tree (as in ref. 15) is retained in light of ref. 30. However, as a rapidly diverging single isolated sequence, its placement on the tree may need future revision.

increased efficiency, and ruminant artiodactyls have been enormously successful in competition with the herbivorous perissodactyls (for example, horses, tapirs and rhinoceroses), which maintain fermentation in digestion in the caecum following the small intestine.

Barnard proposed over twenty years ago that fore-stomach digestion creates a need for especially large amounts of intestinal RNase²³. Fermenting bacteria deliver large amount of RNA (mRNA, tRNA, rRNA) to the gastric region of the stomach and the small intestine; between 10 and 20% of the nitrogen in

the diet of a typical bovid enters as RNA²³. Consistent with this hypothesis is the fact that fore-stomach digesters have much higher levels of pancreatic RNase than other artiodactyls (such as the pig) and non-ruminant ungulates (such as the horse)²³.

The fact that a ribonuclease emerged with increased stability, decreased catalytic activity against duplex RNA, and increased levels of expression, at the same time as ruminant digestion emerged, may of course be a coincidence; but it may also indicate that ancestor **h** and its predecessors **i** and **j** were not specialized digestive enzymes of the bovine RNase A type, but rather played

TABLE 1 Sequence changes in reconstructed ancient ribonucleases

Bovine RNase A		Ancestral sequences												
		a	b	c	d	e	f	g	h₁	h₂	i₁	i₂	j₁	j₂
3	Thr	Thr	Thr	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser [*]	Thr [*]	Ser	Ser
6	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Glu	Glu	Lys	Lys
15	Ser	Ser	Ser	Ser	Ser	Pro	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
16	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Gly [*]	Gly [*]	Gly [*]	Gly [*]	Gly	Gly
17	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ser [*]	Thr [*]	Ser	Ser	Ser	Ser
19	Ala	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
20	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ser	Ser	Ser	Ser	Ser	Ser
22	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Asn [*]	Asn [*]	Asn	Asn
31	Lys	Lys	Lys	Lys	Lys	Gln	Lys	Lys	Lys	Lys	Lys	Lys	Lys [*]	Lys [*]
32	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Arg	Arg	Arg	Arg
34	Asn	Asn	Asn	Asn	Asn	Asn	Asn	Asn [*]	Lys [*]	Lys [*]	Lys [*]	Lys [*]	Asn	Asn
35	Leu	Met	Met	Leu	Leu	Leu	Leu	Leu [*]	Met	Met	Met	Met	Met	Met
37	Lys	Lys	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln	Gln
38	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Asp	Gly	Gly	Gly	Gly	Gly	Gly
59	Ser	Ser	Ser	Ser	Ser	Phe	Ser	Ser	Ser	Ser	Ser	Ser	Ser	Ser
64	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Thr	Thr	Thr	Thr	Thr	Thr
70	Thr	Thr	Thr	Thr	Thr	Ser	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
76	Tyr	Tyr	Tyr	Tyr	Tyr	Asn	Tyr	Asn	Asn	Asn	Asn	Asn	Asn	Asn
78	Thr	Thr	Thr	Thr	Thr	Ala	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr
80	Ser	Ser	Ser	Ser	Ser	His	Ser	Arg [*]	Arg [*]	Arg [*]	His	His	His	His
96	Ala	Ala	Ala	Ala	Ala	Val	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala
100	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Thr	Ser	Ser	Ser	Ser
102	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Ala	Val	Val	Val [*]	Val [*]	Val [*]	Glu [*]
103	Asn	Lys	Lys	Lys	Glu	Glu	Glu	Glu	Glu	Glu	Gln	Gln	Gln	Gln

Reconstructed ancient sequences are designated by lower-case bold letters. The ancient sequences were adapted from Beintema *et al.*¹⁵, calculated using the maximum parsimony procedure of Fitch² at the amino-acid level. A single modification in the Beintema *et al.* tree (Fig. 1 legend) resolved several ambiguities in the Beintema-Fitch reconstructions without altering any unambiguously assigned amino acids. Those amino acids marked with an asterisk indicate positions in which assignment depends on ambiguous parsimony reconstructions, or might be changed by plausible reorganization of the tree. In several of these cases, multiple sequences were reconstructed; subscripts indicate alternative sequence reconstructions for one node in the tree.

TABLE 2 Kinetic properties of reconstructed ancestral ribonucleases

RNase	Ancestor of	k_{cat}/K_m UpA $\times 10^6$	k_{cat}/K_m as % of RNase A	Poly(U) relative to RNase A	Poly(A):poly(U) relative to RNase A
RNase A*		5.0	100	100	1.0
RNase A†		4.5	90	97	1.0
a	ox, buffalo, eland	6.1	122	106	1.4
b	ox, buffalo, eland, nilgai	5.9	118	112	1.0
c	b and the gazelles	4.5	91	97	0.8
d	Bovids	3.9	78	86	0.9
e	Deer	3.6	73	77	1.0
f	Deer, pronghorn, giraffe	3.3	67	103	1.0
g	Pecora	4.6	94	87	1.0
h₁	Pecora and seminal RNase	5.5	111	106	5.2
h₂	Pecora and seminal RNase	6.5	130	106	5.2
i₁	Ruminata	4.5	90	96	5.0
i₂	Ruminata	5.2	104	80	4.3
j₁	Artiodactyla	3.7	74	73	4.6
j₂	Artiodactyla	3.3	66	51	2.7

RNase names refer to nodes in the evolutionary tree shown in Fig. 1. All assays were performed at 25 °C. For UpA (Sigma), kinetic values were determined in 100 mM sodium acetate (pH 5.0). For poly(U), kinetic values were determined in 10 mM sodium acetate (pH 5.0) containing 150 NaCl and 20 $\mu\text{g ml}^{-1}$ substrate following change in absorbance at 260 nm over a period of 90 s, using 200–250 ng of RNase per assay. For poly(A) : poly(U) (made by mixing poly(A) and poly(U) from Boehringer Mannheim in equimolar amounts and preannealing)²⁵, kinetic values were determined in 10 mM Tris-HCl (pH 7.3) containing 150 mM NaCl, 2 mM MgCl_2 , and 30 $\mu\text{g ml}^{-1}$ substrate following change in absorbance at 260 nm over a period of 5 min, using 1–2 μg RNase per assay²⁹.

* Expressed in *E. coli*.

† From Boehringer Mannheim.

a non-digestive role. Of relevance to this hypothesis is the divergence of two non-digestive members of the RNase superfamily at approximately this point on the tree, RNase from brain²⁴ and RNase from seminal plasma. The physiological significance of catalytic activity against duplex RNA in non-digestive RNases is not yet known. It is interesting to note, however, that bovine seminal RNase has still higher catalytic activity against duplex RNA²⁵.

Unfortunately, the connectivity of deep branches in the evolutionary tree is not fully specified, either by sequence data or by fossil records, and remains disputed (Table 1)²⁶. This makes conclusions that might be drawn from these experiments alone insecure. Therefore, we explored the structural origin of the increased catalytic activity of the ancestral RNases by further site-directed mutagenesis experiments. We found that a variant of **h₁** that restores aspartic acid at position 38 (as in RNase A) has a catalytic activity against duplex RNA similar to that of RNase A²⁷. Conversely, a variant of RNase A that introduces Gly alone at position 38 has catalytic activity against duplex RNA which is essentially that of ancestor **h**. These results show

TABLE 3 Thermal transition temperatures for reconstructed ancient ribonucleases

Enzyme	T_m °C	ΔT_m °C
RNase A*	59.3	0.0
RNase A†	59.7	+0.4
a	60.6	+1.3
b	61.0	+1.7
c	60.7	+1.4
d	58.4	-0.9
e	61.1	+1.8
f	58.6	-0.7
g	59.1	-0.2
h₁	58.9	-0.5
h₂	59.3	0.0
i₁	58.2	-1.1
i₂	58.7	-0.6
j₁	56.5	-2.8
j₂	57.1	-2.2

Melting temperatures (± 0.5 °C) were determined according to ref. 21 in 100 mM sodium acetate (pH 5.0).

* Expressed in *E. coli*.

† From Boehringer Mannheim.

that substitution at a single position (residue 38) accounts for essentially all of the increased catalytic activity against duplex RNA in ancestor **h**.

As shown in Fig. 1, the reconstructed amino acids at position 38 are unambiguous throughout the tree, even at the most ancient nodes. Thus, it is highly probable that a change in catalytic activity against duplex RNA in fact occurred in RNases at this point. In one interpretation, catalytic activity against duplex RNA was not necessary in the descendent RNases, and therefore was lost. This implies that the replacement of Gly 38 by Asp in the evolution of ancestor **g** from ancestor **h** was neutral. We cannot, however, rule out an alternative model, that Asp 38 confers positive selective advantage on RNases found in advanced ruminants, an interpretation similar to that used to interpret the evolution of lysozymes in ruminants and their evolutionary analogues²². Interestingly, an Asp is present at position 38 both in many true ruminants and in the hippopotamus. Although the hippopotamus is not a true ruminant (it does not chew its cud), it does have a complex fore-stomach similar to that found in true ruminants²⁸. This suggests the intriguing possibility that this substitution may have an adaptive function in RNases in organisms that have foregut digestion. In either case, these experiments show the value of parsimony analysis as a source of inspiration in experimental biochemistry and as a tool for understanding the physiological role of proteins better, and should encourage a more widespread use of evolutionary reconstruction as an experimental tool to guide site-directed mutagenesis. □

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Defective myosin VIIA gene responsible for Usher syndrome type 1B

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USHER syndrome represents the association of a hearing impairment with retinitis pigmentosa¹ and is the most frequent cause of deaf-blindness in humans. It is inherited as an autosomal recessive trait which is clinically and genetically heterogeneous^{2,3}. Some patients show abnormal organization of microtubules in the axoneme of their photoreceptors cells (connecting cilium)^{4,6}, nasal ciliary cells⁷ and sperm cells⁵, as well as widespread degeneration of the organ of Corti⁸. Usher syndrome type 1 (USH1) is characterized by a profound congenital sensorineural hearing loss, constant vestibular dysfunction and prepubertal onset of retinitis pigmentosa. Of three different genes responsible for USH1⁹⁻¹¹, *USH1B* maps to 11q13.5 (ref. 10) and accounts for about 75% of USH1 patients^{2,3}. The mouse deafness *shaker-1* (*sh1*) mutation has been localized to the homologous murine region^{12,13}. Taking into account the cytoskeletal abnormalities in USH patients, the identification of a gene encoding an unconventional myosin as a candidate for *shaker-1* (ref. 14) led us to consider the human homologue as a good candidate for the gene that is defective in USH1B. Here we present evidence that a gene encoding myosin VIIA is responsible for USH1B. Two different premature stop codons, a six-base-pair deletion and two different missense mutations were detected in five unrelated families. In one of these families, the mutations were identified in both alleles. These mutations, which are located at the amino-terminal end of the motor domain of the protein, are likely to result in the absence of a functional protein. Thus USH1B appears as a primary cytoskeletal protein defect. These results implicate the genes encoding other unconventional myosins and their interacting proteins as candidates for other genetic forms of Usher syndrome.

A lambda subclone from YAC 965 (encompassing the *USH1B* locus¹⁵) was found to contain a 4.2-kilobase (kb) *EcoRI* fragment which hybridized to a 2-kb mouse genomic fragment comprising five exons of a myosin gene¹⁴. We identified ten exons by sequencing this fragment and the immediately adjacent 6-kb

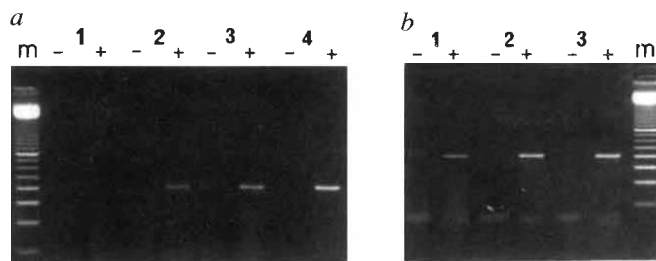


FIG. 1 RT-PCR analysis of myosin VIIA expression in human (a) and mouse (b) tissues. a, A 402-bp specific product was detected in liver (lane 2), kidney (lane 3) and retina (lane 4) but not in brain (lane 1). b, A 407-bp product is detected in retina (lane 1), cochlea (lane 2) and kidney (lane 3). The plus and minus signs indicate that the PCR reaction was done with or without reverse transcriptase. Lane m shows a 100-base ladder (BRL).

METHODS. Total RNA was transcribed using primer 1 (overlapping the junction between the fourth and fifth exons) and then amplified for 40 cycles with primer 1 and primer 2 (overlapping the first and second exons). Primer sequences were: human primer 1, 5'-TATGCAGT-TACCCATGGCCAAG-3'; human 2, 5'-CTGCATCATCAGTGGGAATC-3'; mouse primer 1, 5'-CAGGTTGATGCAGTACCAGT-3'; mouse 2, 5'-CTGTATTATCAGCGGGGAAG-3'.

EcoRI fragment. Subsequent isolation of clones from a retinal complementary DNA library extended the sequence to 1,851 base pairs (bp) (NCBI accession number, U17180). The deduced amino-acid sequence encodes most of the motor head of myosin and is 96% identical to murine myosin. The human sequence includes a previously characterized sequence of 31 residues, referred to as myosin VIIA (ref. 16).

Expression of the myosin VIIA gene was studied using the reverse-transcription polymerase chain reaction (RT-PCR) technique. RT-PCR products of the expected sizes were detected in human kidney, liver and retina, but no RT-PCR product was seen in brain or in lymphocytes transformed by Epstein-Barr virus (Fig. 1a); in the mouse, expression was detected in the retina, cochlea, kidney and liver (Fig. 1b). The sequences of the PCR products matched those of the isolated cDNAs.

A search for mutations was undertaken in nine unrelated families³ in which segregation analysis indicated that the *USH1B* gene could be involved. The five 5'-most exons were amplified using intronic flanking primers and the PCR products sequenced. In three families, mutations were detected in four exons, confirmed by sequencing of independent PCR products subcloned in M13 vectors. In family 1, a heterozygous C→T transition in the first exon (nucleotide 163), resulting in a premature stop codon, was found in the two affected probands and their mother, but not in their unaffected brother and father (Fig. 2a). In family 2, a heterozygous C→T transition in the third exon (nucleotide 108), also resulting in a premature stop codon, was detected in the affected proband and his mother but not in his unaffected brother and father (Fig. 2b). In families 1 and 3, the same in-frame 6-base-pair (bp) deletion (GACATC) was observed in exon 3 (position 60 to 65), leading to loss of amino-acid residues D and I. In family 1, the two affected brothers and their father were heterozygous for the deletion. In family 3, the two affected probands and their mother, but not their father, carried the deleted allele (Fig. 2b). The finding of the same mutation in families originating from different geographical regions suggests that two independent mutational events have occurred, indicating a propensity of this site to mutation. Because this hexanucleotide deletion occurs in an 11-bp sequence containing two 5-bp direct repeats, it is tempting to speculate that either replication slippage or slipped-strand mispairing is responsible for the mutational event. In another series of ten families², two different heterozygous missense mutations in exon 3 were detected in two families at position 42 (C→T in family A) and position 43 (G→A in family B) (Fig. 2b). These mutations

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respectively substitute a cysteine and a histidine codon for the same arginine codon. None of these mutations was found in the 74 alleles from the general population that were sequenced as controls.

The identification of the mutations in both alleles of the affected children from family 1, and the cosegregation of each of these mutations with the disease, provide strong evidence that the myosin VIIA gene is responsible for USH1B. Moreover, the nonsense mutations found in exons 1 and 3 would result in proteins truncated before the ATP-binding site and the actin-binding site respectively and therefore not functional. The isoleucine present in the two-amino-acid deletion is a highly conserved residue in conventional (myosin II) as well as unconventional myosins (Fig. 3). These two residues are located inside a large β -sheet¹⁷ and hence are likely to result in an incorrect folding of the motor domain and consequently in an inactive protein. The two substitutions of the arginine present in a highly conserved heptapeptide sequence (Fig. 3) close to the phosphate-binding site¹⁷ would also be expected to impair the function of this myosin.

Our results and those described in the accompanying Letter establish that *USH1B* and *shaker-1* involve the orthologous myosin VIIA genes. But the phenotypes of the original *sh1* mutant and of the five other *sh1* mutants seem to be different from that of USH1 patients, because no sign of retinal degeneration has so far been reported in these mouse mutants^{18,19}. Therefore either another myosin could compensate for a defective myosin VIIA in the mouse retina or the nature of the mutation itself¹⁴ might account for the phenotypic difference. One form of human neurosensory recessive deafness without retinal dystrophy, attributed to the gene *DFNB2*, which maps to the same chromo-

Myosin I consensus ³⁰	: FGNAKT.RNNSSSRFGK ^Y mEIqFD
Myosin II consensus ³⁰	: FGNAKT ^v RnN ^{SSSR} FGK ^F IrIhF.
Myosin VI mouse ³¹	: IGNAKTTRND ^{SSSR} FGK ^Y IEIGFD
Myosin VI human, pig ¹⁶	: FGNAKT ^v RnN ^{SSSR} FGK ^F VEIhFN
Human myosin VIIA	: FGNAKTIRND ^{SSSR} FGK ^Y IDIHFN

FIG. 3 Amino-acid alignments in different myosins around the missense mutations and the 2-amino-acid deletion. The heptapeptide sequences containing the mutated arginine and the deleted isoleucine are indicated in bold.

somal region as *USH1B*, may represent the human equivalent of the *sh-1* mutants¹⁵.

Unconventional myosins are motor molecules with structurally conserved heads, which move along actin filaments using their actin-activated ATPase activity. Their highly divergent tails are presumed to be tethered to different macromolecular structures that move relative to actin filaments^{20,21}. If we assume that the anomalous connecting cilium of photoreceptor cells described in some USH patients is associated with clinical subtype 1B, we have to postulate an interaction between actin filaments and microtubules involving myosin VIIA. The presence of myosin at the base of connecting cilia²² is consistent with this postulate. According to this hypothesis, the kinocilium of cochlear and vestibular hair cells could be the primary defective structure in the inner ear²³. However, such an interaction between microfilaments and the microtubule network is still poorly documented^{24,25}. Alternatively, in the absence of direct evidence for abnormal microtubular structures in USH1B, a role for myosin VIIA is a possibility in the microvilli of the retinal pigment cells, the inner-ear supporting cells and in the hair-cell stereocilia. Such a role is consistent with the presence of unconventional myosins in intestinal cell microvilli²⁶ and at the tip of the hair-cell stereocilia, where one or several myosins are presumably responsible for modulating the tip-link tension that controls the gating of cation-transduction channels²⁷⁻²⁹.

As there are at least seven genes responsible for Usher syndrome², it is possible that abnormalities in the genes encoding other unconventional myosins and their various binding components are responsible for other genetic forms of this dual sensory defect. □

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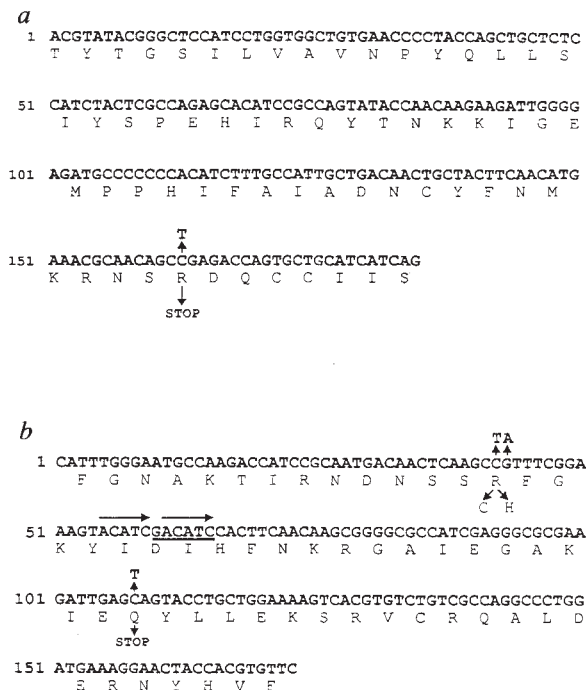


FIG. 2 Nucleotide and deduced amino-acid sequences of the two myosin VIIA exons of the human gene containing the mutations. **a**, Exon 1; **b**, exon 3. The mutations are indicated above and below the normal sequences and the 6-bp deletion which was found in two different families is underlined. Arrows show the 5-bp direct repeat. A silent polymorphism was detected in exon 1 at the third position (G→A) and in exon 3 at position 83 (C→T). The primers used for sequencing were: 1a, 5'-ACTAACTCCGAGCCAGAC-3'; 1b, 5'-GTTACACAGCAGAGTACAT-AG-3'; 3a, 5'-GATTGAGCAGCAGGCTT-3'; 3b, 5'-CAATACGGGCAGCAAT-AC-3'.

A type VII myosin encoded by the mouse deafness gene *shaker-1*

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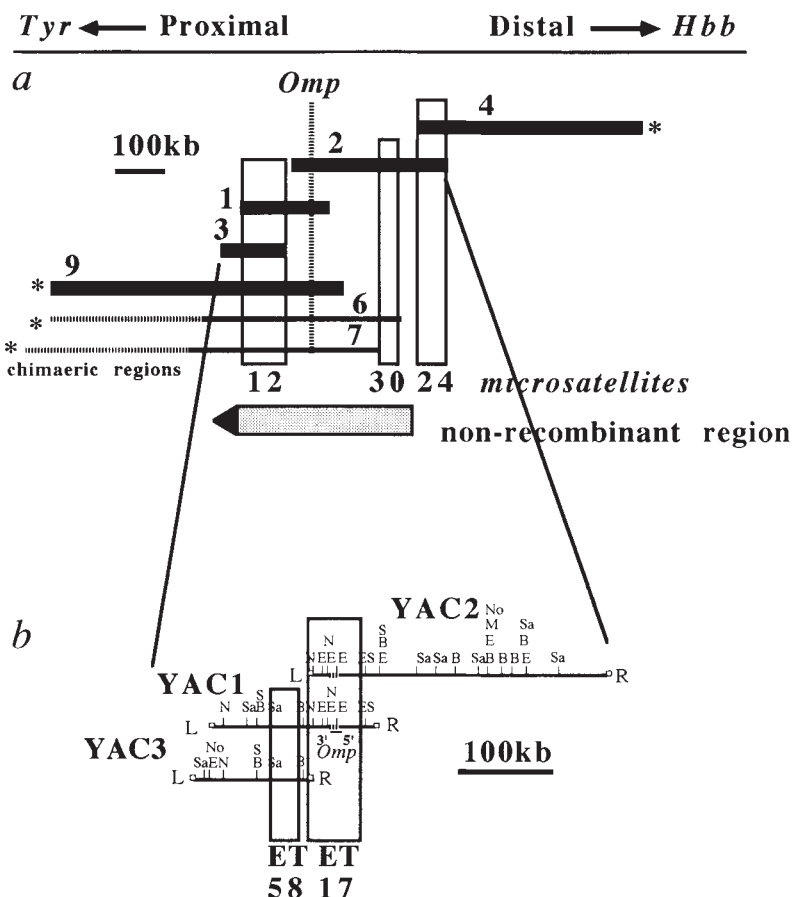
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GENETIC deafness is common, affecting about 1 in 2,000 births¹. Many of these show primary abnormalities of the sensory neuroepithelia of the inner ear, as do several hearing-impaired mouse mutants, suggesting that genes involved in sensory transduction could be affected. Here we report the identification of one such gene, the mouse *shaker-1* (*sh1*) gene. *Shaker-1* homozygotes show hyperactivity, head-tossing and circling due to vestibular dysfunction, together with typical neuroepithelial-type cochlear defects

involving dysfunction and progressive degeneration of the organ of Corti²⁻⁷. The *sh1* gene encodes an unconventional myosin molecule of the type VII family. Three mutations are described, two missense mutations and a splice acceptor site mutation, all in the region encoding the myosin head. The myosin type VII molecule encoded by *sh1* is the first molecule to be identified that is known, by virtue of its mutations, to be involved in auditory transduction.

The Olfactory marker protein gene (*Omp*) is very tightly linked to the mouse *sh1* mutation on mouse chromosome 7 (ref. 8). In a backcross of 1,066 progeny segregating for the *sh1* mutation, *Omp* is non-recombinant with *sh1*, indicating that the *sh1* locus is likely to lie within 0.1 centimorgan (~200 kilobases) of the *Omp* gene. *Omp* was used as a start point for the construction of a yeast artificial chromosome (YAC) contig across the *sh1* region (Fig. 1). Two YAC clones (YACs 1 and 2) containing *Omp* were isolated and used to extend the contig both distally and proximally. Microsatellites isolated from YACs 1 and 2 were used to rescreen mouse YAC libraries to isolate extending YAC clones. These markers were also used to analyse backcross progeny from the *sh1* cross to identify the closest recombination breakpoints flanking the *sh1* locus and thus narrow the search for the *sh1* gene. A tiling path of five non-chimaeric YACs (YACs 9, 3, 1, 2 and 4) was identified that spans a 1.4-megabase region around *Omp* (Fig. 1). Microsatellites 12 and 30 were non-recombinant with *sh1*, whereas microsatellite 24 detected one recombinant with *sh1* delimiting the non-recombinant region around *sh1* to the end of YAC 2 (Fig. 1), orientating the contig

FIG. 1 Gene identification of the *sh1* region of mouse chromosome 7. **a**, A YAC contig of seven clones was established over a 1.4-Mb region surrounding the *Omp* gene by analysis of three mouse YAC libraries available for PCR-based screening (the Princeton²⁴, St Mary's²⁵ and MIT²⁶ mouse YAC libraries). Internal microsatellites were isolated from the first YACs identified (YACs 1 and 2) by gel purification of YAC DNA by pulsed-field gel electrophoresis⁹, digestion with *Sau3A* and cloning into pBluescript followed by colony screening with a CA oligonucleotide. After sequencing, primers were designed for microsatellites 12, 24 and 30: 12: TGGTACGGATCTGACGAGACAA/GGTGAGTGCAGCCTGTCTAGC; 24: ACTGTGCCAGCTTCTTCTCAAT/TTGGGTGTTTCTGTGTCATGTTCC; 30: GGAAGCCTTTATGAATTATTACC/CTAAGCTGATTGAGTGTGAAATCAT. Microsatellites 12, 24 and 30 were used to confirm the internal integrity of the contig in the region of YAC 1 and YAC 2 as well as extend the contig by the isolation of YACs 4, 6, 7 and 9 by PCR screening of the available mouse YAC libraries. Simple sequence-length polymorphisms (SSLPs) for microsatellites 12, 24 and 30 were identified between the parental strains of the *sh1* backcross (CBA/Cash1/*sh1* and C57BL/10; ref. 8). SSLP segregation analysis of microsatellites 12, 24 and 30 through the *sh1* backcross recombinant panel⁸ was used to confirm their location on chromosome 7 and to locate flanking breakpoints to the *sh1* gene. Furthermore, unique end clones (indicated by asterisks) were also isolated from the extending YACs 4, 6, 7 and 9 by the pICL/pLUS vector system²⁷. Subsequently, restriction-fragment length variants for these end clones were identified in either the parental strains for the *sh1* backcross or in a second interspecific backcross segregating markers on mouse chromosome 7 (ref. 28). Segregation analysis through either of these crosses was used to assess the location of end clones to chromosome 7. Clones 4 and 9 were non-chimaeric; clones 6 and 7 were chimaeric at their proximal ends. In addition, we demonstrated that the distal end clone of YAC 4 was also recombinant in the same backcross mouse as microsatellite 24, confirming that the distal limits to the non-recombinant region had been identified. **b**, YAC restriction maps in the vicinity of *Omp* and the identification and mapping of new genes in this region. B, *Bss*HI; E, *Eag*I; M, *Mlu*I; S, *Sst*II; Sa, *Sa*I; No, *Not*I. Note a presumed polymorphism between YAC 1 and YAC 3 for a *Sa*I site: YAC 1 and YAC 3 were obtained from the



Princeton²⁴ and St Mary's²⁵ YAC libraries, respectively, which were derived from different mouse strains. Exon trapping of YAC 1 was carried out as described⁹ and identified two conserved exons, ET17 and ET58, which were shown by hybridization analysis to map to the indicated regions of YAC 1 close to *Omp* and to the expected overlapping regions of YAC 2 and YAC 3, respectively.

with respect to the flanking proximal and distal markers and identifying a non-recombinant region of ~500 kilobases (kb) stretching over YAC 1 and YAC 2. Although we had not determined the proximal limits of the non-recombinant region, we began a search for potential candidate genes in this 500-kb region by exon trapping.

Nine unique exon-trap products were recovered from the 175-kb YAC 1, of which two (ET17 and ET58) contained conserved sequences and mapped to different regions of the YAC (Fig. 1 and ref. 9). ET58 was used to isolate a 4.6-kb clone from a mouse inner-ear complementary DNA library (Fig. 2 legend). Sequence analysis of the 4.6-kb cDNA indicated that it encoded a myosin-like protein; however, this cDNA was unspliced and contained only 795 base pairs (bp) of coding sequence from the myosin head region, which comprised five complete exons, starting with ET58 at the 5' end, and a portion of a sixth exon truncated by the cloning site at the extreme 3' end. Nevertheless, the available myosin sequence indicated that ET58 encoded a myosin type VII, an unconventional family of myosin molecules¹⁰, whose tail sequence and structure are unknown but for which porcine, human and frog sequences from portions of the head region are available^{11,12}. Comparison of protein sequence just C-terminal of the ATP-binding domain in this relatively diverged region with motifs that define the other myosin classes¹¹ demonstrated 100% identity with pig myosin

TABLE 1 *Shaker-1* mutations identified in the myosin VII gene

Mutant allele	Origin	Mutation type	Sequence change
<i>sh1</i>	Spontaneous	Missense; Arg→Pro	AAC CGG CCT C
<i>sh1^{6J}</i>	Spontaneous	Missense; Arg→Pro	TCA CGT GTC C
<i>sh1^{26SB}</i>	ENU	3' splice site 30-bp in-frame deletion + cryptic splice products	cag CTC TTC
<i>sh1^{816SB}</i>	ENU		g
<i>sh1^{3336SB}</i>	ENU		
<i>sh1^{4494SB}</i>	ENU		
<i>sh1^{4626SB}</i>	ENU		

For each *sh1* mutation, the appropriate background strain was also sequenced. *sh1* arose on a BALB/c background². *sh1^{6J}* is a spontaneous mutation which arose on the inbred C57BL/KsJ-*dbm* strain¹⁴ and is coisogenic with this strain. *sh1^{26SB}*, *sh1^{816SB}*, *sh1^{3336SB}*, *sh1^{4494SB}* and *sh1^{4626SB}* ENU mutants¹⁵ were all derived in BALB/cR1 mice. For the ENU-induced mutations, apart from sequencing the background strain, the five alleles also act as a set of internal controls.

VIIA and 97% identity with human myosin VIIA classes (Fig. 2). Subsequent careful comparison of the remaining exon traps from YAC 1 with myosin sequences in the database indicated that two further exons, ET29 and ET44, were derived from

FIG. 2 Amino-acid sequence of head region of mouse myosin VII gene. The positions of detected mutations in *sh1* alleles are indicated (Table 1): *sh1*, arginine-to-proline change at position 407 (indicated in bold); *sh1^{6J}*, arginine-to-proline change at position 145 (indicated in bold); and *sh1^{816SB}*, at positions 551–560 (the 10-amino-acid deletion is struck through). In addition, the exon structure is indicated where known (adjacent exons are indicated by alternative double and thick underlining). Five complete exons have been characterized together with part of a sixth exon to position 265 (dotted underlined). The ATP-binding and actin-binding sites of the myosin head are also shown, as are the characteristic sequence motifs for human and pig myosin VII (ref. 11), which are aligned with the mouse myosin sequence.

METHODS. Myosin head sequence was obtained from a 4.6-kb cDNA homologous to ET58 which was derived by screening a mouse inner-ear cDNA library (K.W.B. unpublished results). This unidirectional cDNA library was constructed using the λ ZAP XR vector (Stratagene). The primary library contained 1.05×10^5 phage and the amplified library had a titre of 9.1×10^9 PFU ml⁻¹, with 97.7% being recombinants with an average insert size of 1.1 kb. The 4.6-kb cDNA was unspliced and following sequencing allowed us to identify five complete exons and part of a sixth exon (see text) in the N-terminal region of the head. Further head sequence was recovered by RT-PCR using primers developed to exon-trap product ET44. An RT-PCR product of ~2 kb, spanning ET58 to ET44, was recovered from kidney RNA, cloned into the PCRII cloning vector (Invitrogen) and sequenced. *Pfu* polymerase was used for RT-PCR to ensure maximum fidelity. Further sets of primers were designed from the available head sequence for RT-PCR of kidney RNA from the *sh1* mutants. RT-PCR products were cloned into PCRII and multiple clones sequenced for each RT-PCR product. In the case of *sh1^{6J}*, the mutation was confirmed by identification of a restriction site change associated with the mutation, followed by restriction

ET58

1 TYTGSILVAV NPYQLLSIYS PEHIRQYTNK KIGEMPPHIF AIADNCYFNM KRNNRDOCCI

ATP-bind

61 IRGESGAGKT ESTKLILQFL AAISGQHSWI EQQVLEATPI LEAFNGAKTI RDNSSRFKG

human VIIA ESTKLIPQFL AAISGQHSWI EQQVLEATPI L

pig VIIA ESTKLILQFL AAISGQHSWI EQQVLEATPI L

P *sh1^{6J}*

121 YIDIHFNKRK AIEGAKIEQY LLEKSRVCRQ APDERNYHVF YCMLEGMNEE EKKKLGLGQA

181 ADNYLAMGN CITCEGRVDS QEYANIRSAM KVLMTDTEN WEISKLLAAI LHMGNLOYEA

241 RTFENLDACE VLFSPSLATA ASLLEVNPPD LMSCLTSRTL ITRGETVSTP LSREQALDVR

301 DAFVKGIYGR LFVWIVEKIN AAIYKPPPLE VKNSRRSIGL LDIFGFENFT VNSFEQLCIN

P *sh1*

361 FANEHLQQFF VRHVFKEQE EYDLESIDWL HIEFTDNQEA LDMIANRPMN VISLIDEESK

421 FPKGTDATML HKLNSQHKLN ANYVPPKNSH ETQFGINHFA GVVYYESQGF LEKNRDTLHG

Actin-binding

481 DIQLVHSSR NKFKVQIFQA DVAMGAETRK RSPTLSSQFK RSELELMRTL GACQFFVRC

sh1^{816SB}

541 IKPNEFKKPM LFDRLHLCVRQ LRYSGMMETI RIRHAGYPIR YSFVEFVERY RVLLPGVKPA

ET44

601 YKQDGLRGTC ORMAEAVLGT HDDWOIGKTK I

enzyme digestion analysis of RT-PCR products of *sh1^{6J}* and the coisogenic C57BL/KsJ-*dbm* background strain. An *Afl*III site is destroyed in *sh1^{6J}*.

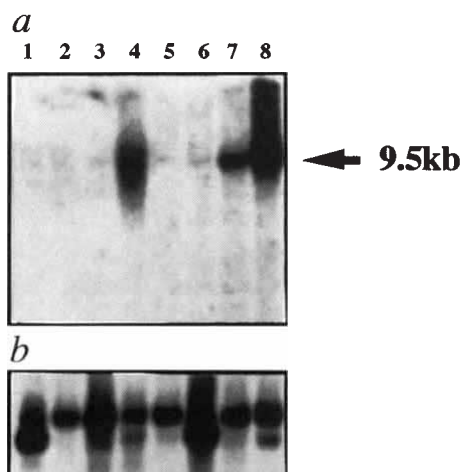


FIG. 3 Expression of the mouse myosin VII gene. *a*, Northern analysis of the expression of mouse myosin VII in a variety of mouse tissues. Lanes 1, heart; 2, brain; 3, spleen; 4, lung; 5, liver; 6, skeletal muscle; 7, kidney; 8, testis. *b*, Filters rehybridized with a β -actin probe to assess RNA loading.

METHODS. A 4.6-kb cDNA detected from a mouse inner-ear library and containing exons from the myosin VII gene, was hybridized to a Multiple Tissue Northern (Clontech) with 2 μ g poly(A)⁺ RNA from adult mouse tissues. Hybridizations were done in 5 $\times$ SSPE, 10 $\times$ Denhardt's solution, 100 μ g ml⁻¹ salmon sperm DNA, 50% formamide, 2% SDS, with 5 $\times$ 10⁶ c.p.m. probe per ml hybridization solution. Filters were washed to 0.5 $\times$ SSC at 55 $^{\circ}$ C and exposed for 1 week.

myosin-like sequences and appeared to be part of the same myosin VII gene derived from the C-terminal region of the myosin head. Reverse-transcription polymerase chain reaction (RT-PCR) between ET44 and ET58 produced the expected product of ~2 kb and allowed us to recover the bulk of the head-region sequence encompassing 1,893 bp, including the ATP-binding and actin-binding domains (Fig. 2). Northern analysis detected a myosin VII transcript of ~9.5 kb in lung, kidney and testis (Fig. 3). A very low level of expression was found in skeletal muscle, heart, brain, spleen and liver. In addition, PCR of the ET58 exon from a rat outer-hair-cell cDNA library gave a product of the expected size (data not shown); in the accompanying paper¹³ it is shown that this myosin VII gene is transcribed in mouse cochlea.

All seven available alleles^{2,14,15} at the *sh1* locus were screened for mutations in the myosin VII gene. Three confirmed mutations are detailed in Table 1. RT-PCR products covering the available coding sequence have been recovered and sequenced for all seven mutations, together with the appropriate background strains (Table 1). The *sh1* mutation shows a non-conservative arginine-to-proline change. The *sh1*^{6J} mutation, when compared to the coisogenic background strain C57BL/KsJ-*dbm* strain, shows a non-conservative arginine-to-proline change close to the ATP-binding site of the myosin head. The *sh1*^{6J} mutation was confirmed by restriction analysis (Fig. 2 legend). The altered arginine residue in *sh1*^{6J} is highly conserved and is found in all myosin genes studied so far^{10,12}. Interestingly, an arginine-to-cysteine mutation at this position in *Caenorhabditis elegans* skeletal muscle myosin shows a slow-moving phenotype and some thick-filament disorganization¹⁶. In the mutation *sh1*^{816SB}, which was induced by *N*-ethyl-*N*-nitrosourea, abnormal RT-PCR products were observed using primers in the region of the actin-binding site, indicating a possible splice-site mutation. Instead of the normal 215-bp PCR product, a smaller one of 185 bp and two larger ones of 279 bp and 318 bp were observed (data not shown). The 185-bp PCR product resulted from an in-frame deletion of ten codons just four codons downstream of the actin-binding site (Fig. 2). The larger PCR products, as expected, contained intron sequence upstream of the deleted ten codons and enabled us to identify a 3' splice-site mutation (Table 1) which presumably leads to exon skipping and the observed in-frame 30-bp deletion. The larger PCR products presumably arise through upstream cryptic splice sites in the intron; sequencing of the two larger products demonstrated that both would result in premature termination (data not shown).

We have identified the molecular basis for an autosomal recessive deafness phenotype in the mouse. A myosin molecule plays a pivotal role in the adaptation motor^{17,18}, which is thought to be responsible for adjusting the tip-link tension between

stereocilia that controls the gating mechanism of mechanotransduction in the hair cell. There is evidence to implicate myosin I (ref. 17), but given the direct involvement in hearing demonstrated here for myosin VII, we propose that this molecule is also an excellent candidate for the adaptation motor. The involvement of a myosin molecule in deafness also suggests a new class of candidate genes for other deafness loci. The *sh1* gene maps to a region of mouse chromosome 7 that is homologous to human 11q13, to which the *Usher 1b* (*USH1B*) locus maps^{19,20}. Usher syndrome type 1 is an autosomal recessive syndrome which includes severe congenital hearing impairment and vestibular dysfunction but is also associated with slowly progressive retinitis pigmentosa. Three loci are thought to be involved at 11q (refs 19, 20), 11p (ref. 21) and 14q (ref. 22). In addition, a non-syndromic autosomal recessive locus (*DFNB2*) has been assigned to 11q13 (ref. 23). In the accompanying paper, it is shown that the myosin VII gene that underlies *sh1* also underlies *USH1B* (ref. 13). □

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Abnormal avoidance learning in mice lacking functional high-affinity nicotine receptor in the brain

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NICOTINE affects many aspects of behaviour including learning and memory^{1,2} through its interaction with neuronal nicotinic acetylcholine receptors (nAChR). Functional nAChRs are pentameric proteins containing at least one type of α -subunit and one type of β -subunit³⁻⁵. The involvement of a particular neuronal nicotinic subunit in pharmacology and behaviour was examined using gene targeting to mutate $\beta 2$, the most widely expressed nAChR subunit in the central nervous system⁶⁻⁸. We report here that high-affinity binding sites for nicotine are absent from the brains of mice homozygous for the $\beta 2$ -subunit mutation. Further, electrophysiological recording from brain slices reveals that thalamic neurons from these mice do not respond to nicotine application. Finally, behav-

ioural tests demonstrate that nicotine no longer augments the performance of $\beta 2^{-/-}$ mice on passive avoidance, a test of associative memory. Paradoxically, mutant mice are able to perform better than their non-mutant siblings on this task.

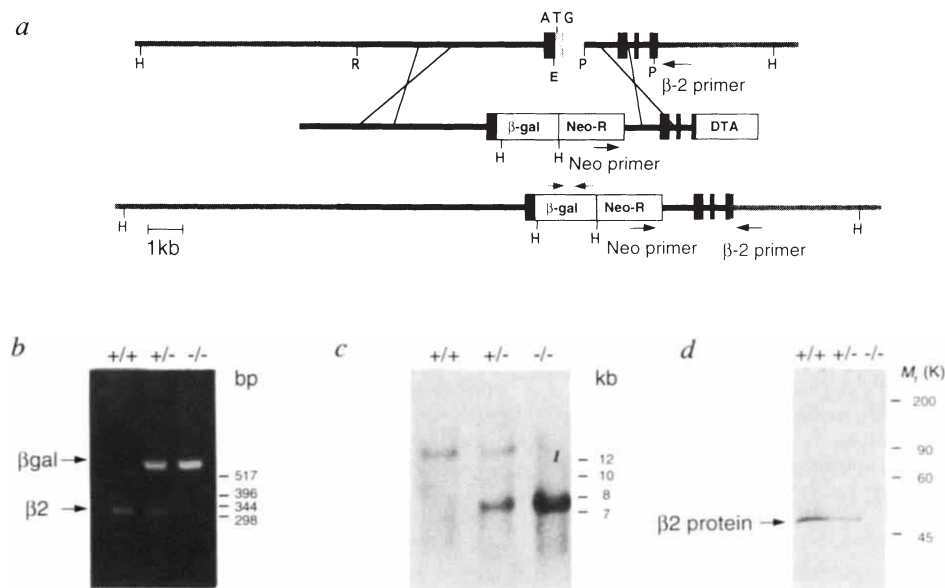
The $\beta 2$ -subunit of the nAChR was disrupted in embryonic stem (ES) cells, and mice deficient in this subunit were then generated (Fig. 1). $\beta 2^{-/-}$ mice were viable, mated normally and showed no obvious physical deficits. Overall brain size and organization were normal (see for example Fig. 2a, b). Western blot analysis of total brain homogenates using anti- $\beta 2$ monoclonal 270 (ref. 9; Fig. 1d) and immunocytochemistry throughout the brain using a polyclonal anti- $\beta 2$ antibody⁷ (not shown) demonstrated that the immunoreactivity detected in control mice was absent in $\beta 2^{-/-}$ mice and was diminished in $\beta 2^{+/-}$ mice. $\beta 2$ -encoding messenger RNA was undetectable in $\beta 2^{-/-}$ mice by *in situ* hybridization using $\beta 2$ -antisense oligonucleotides (Fig. 2a).

The distributions of the $\alpha 4$ - and $\beta 2$ -subunits largely overlap in the brain, and these subunits are thought to combine to form the predominant nAChR isoform in the central nervous system (CNS)¹⁰. On the basis of oocyte expression experiments¹¹, $\beta 4$ is the only subunit identified thus far that might also be able to form functional heteropentamers with the $\alpha 4$ -subunit. The $\beta 4$ -subunit was expressed in the medial habenula (MHB) and the interpeduncular nucleus (IPN) in the brain of wild-type, $\beta 2^{+/-}$ and $\beta 2^{-/-}$ mice (Fig. 2a). No $\beta 4$ mRNA was seen elsewhere in the brain of mutant mice, indicating that its transcription was not upregulated to replace the $\beta 2$ -subunit. Nor was the expression of the $\alpha 4$ - (Fig. 2a), $\alpha 5$ - or $\beta 3$ -subunit mRNAs (not shown) significantly altered in mutant mice.

Equilibrium binding experiments have shown that nicotine binds to a population of high-affinity sites (dissociation constant K_D near 10 nM^{12,13}) whose distribution tallies well with that of

FIG. 1 Disruption of the gene encoding the $\beta 2$ -subunit of the neuronal nAChR. a, Top, Normal genomic structure of the mouse $\beta 2$ -subunit gene. Portion of exon one removed by the recombination event is shaded in light grey. ATG-initiator methionine. Boxes represent exons I–IV. Middle, Targeting replacement vector used to disrupt the endogenous $\beta 2$ -subunit gene. Initiator methionine and the rest of the first exon were replaced with the coding region of NLS-*lacZ* and the MC1 *neo^R* expression cassette²³. The construct was able to direct *lacZ* expression after stable transfection of PC12 cells (not shown), but *lacZ* expression was never detected in recombinant animals, despite the lack of obvious recombination in the *lacZ* DNA. Diphtheria toxin-A gene (DTA)²⁴ was used to select against random integration. Bottom, Structure of the mutated $\beta 2$ -gene. Restriction sites: H, *Hind*III; R, *Eco*RI; E, *Eco*47III; P, *Pst*I. Black arrows, primers used to detect recombination events in ES cells. Grey arrows, primers used to detect the wild-type or mutated $\beta 2$ genes. b, PCR analysis of tail DNA from +/+, +/- and -/- mouse. c, Southern blot analysis of tail DNA restricted with *Hind*III from the same mice analysed in b. d, Western blot analysis of total brain protein using a monoclonal antibody raised against the $\beta 2$ -subunit.

METHODS. a, The $\beta 2$ -targeting vector was constructed by inserting a multiple cloning site (MCS) into the MC1 *neo* cassette (GTGACGGTACCGCCCGGCGAGGCTGCTAGCTTAATTAGCGGCGCCTCGAGGGGCCCATGCATGGATCC). A 4.1 kb *Eco*RI–*Eco*47III $\beta 2$ -genomic fragment 5' to the ATG and a 1.5 kb *Pst*I $\beta 2$ -genomic fragment starting within the first intron of the $\beta 2$ -gene were cloned into the MCS. HM1^{25,26}



embryonic stem cells (5×10^7) were transfected with the linearized targeting vector by electroporation as described²³. Twenty-four surviving G418-resistant clones were screened by PCR ($\beta 2$ -primer GCCCAGACAT-AGGTCACATGATGGT; *neo*-primer GTTATTGCAGCTTATAATGGTTACA). Four were positive and were later confirmed by Southern blot analysis (not shown). Clones were injected into 3.5-day-old blastocysts from non-agouti, C57BL/6 mice. All male chimaeric mice were mated to F1, C57BL/6 $\times$ DBA/2 non-agouti females. Of 15 chimaeras, one showed germline transmission. $\beta 2^{+/-}$ heterozygotes were mated and offspring were evaluated by PCR analysis (b). b, PCR was 35 cycles of 94 °C for 1 min, 65 °C for 2 min and 72 °C for 1 min. c, Southern blotting was as described²⁷. The 1.5 kb *Pst*I genomic fragment used for the targeting construct was labelled by random priming. d, Western blotting was as described²⁷ using monoclonal antibody 270⁹.

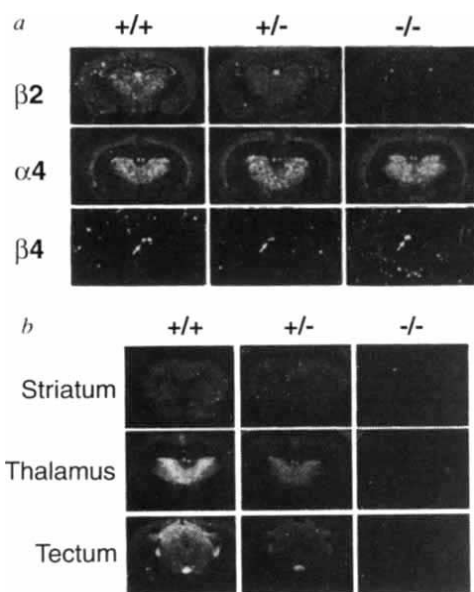


FIG. 2 Mapping of the neuronal nAChR in mouse brain using *in situ* hybridization and tritiated nicotine binding. *a*, *In situ* hybridization using antisense oligonucleotide probes based on the sequence of the cDNAs encoding the $\beta 2$ -, $\alpha 4$ - and $\beta 4$ -subunits of the nAChR to detect their respective mRNAs in serial sections from the brains of $\beta 2^{+/+}$, $\beta 2^{+/-}$ and $\beta 2^{-/-}$ mice. Midthalamic sections are shown. White arrows indicate the MHb labelled by the $\beta 4$ -antisense oligonucleotide. *b*, Receptor autoradiography using tritiated nicotine revealing high-affinity binding sites in the brains of wild-type, heterozygous and $\beta 2$ -mutant mice. Representative sections at the level of the striatum, thalamus and tectum are shown.

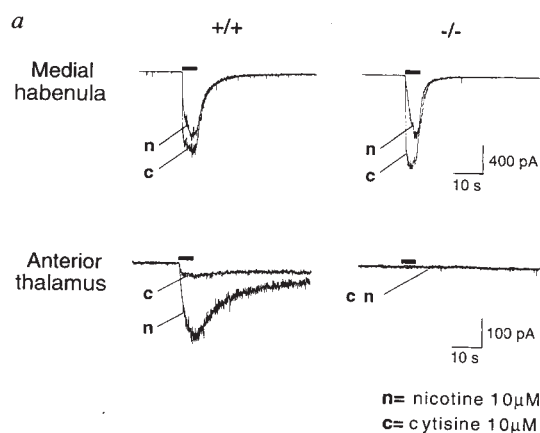
METHODS. *a*, *In situ* hybridization was as described in ref. 8. Oligonucleotides: $\beta 2$, 5'-TCGCATGTGGTCCGCAATGAAGCGTACGCCATCCAC-TGCTTCCCG-3'; $\alpha 4$, 5'-ACCTTCTCAACCTCTGATGTCTTCAAGTCAGGGACCTCAAGGGGG-3'; $\beta 4$, 5'-ACCAGGCTGACTTCAAGACCGGGACGCTTCATG-AAGAGGAAGGTG-3'. *b*, ^3H -nicotine binding was as described in ref. 28. Coronal sections (14 μm) were incubated at room temperature for 30 min in 50 mM Tris pH 7.4, 8 mM CaCl_2 , 4 mM ^3H -L-nicotine. Non-specific binding was evaluated in the presence of 10 μM L-nicotine bitartrate. After incubation, sections were rinsed 2×2 min in ice-cold PBS and briefly rinsed in ice-cold water. Slides were exposed for 60 days to Hyperfilm ^3H .

the $\alpha 4$ - and $\beta 2$ -subunits¹²⁻¹⁴. Quantitative receptor autoradiography was done using ^3H -nicotine (4 nM) to visualize high-affinity nAChR in brain sections from $\beta 2^{+/+}$, $\beta 2^{+/-}$ and $\beta 2^{-/-}$ mice (Fig. 2b). Nicotine binding *in situ* was completely abolished in $\beta 2^{-/-}$ animals, and was reduced by roughly 50% in all brain areas in $\beta 2^{+/-}$ animals, demonstrating that the $\beta 2$ -subunit mediates this high-affinity binding.

Neurons of the anterior thalamus express very high levels of $\beta 2$ - and $\alpha 4$ -subunit mRNAs and responded consistently to 10 μM nicotine in a slice preparation from wild-type animals with an average inward current of 155 ± 73 pA (Fig. 3a) which was blocked by 1 μM dihydro- β -erythroidine. The agonist order of the response was compatible with that seen for $\alpha 4/\beta 2$ -containing nicotine receptors *in vitro*¹¹ (nicotine > DMPP > cytisine) (Fig. 3a).

In $\beta 2^{-/-}$ mice the response of anterior thalamic neurons to nicotine was completely abolished in all neurons tested (Fig. 3b). As a control, neurons in the MHb, where both $\alpha 3$ and $\beta 4$ are strongly expressed, were also tested. Nicotine caused an average inward current of 505 ± 132 pA in wild-type mice, and the agonist potency of this response followed the rank order for the $\alpha 3/\beta 4$ -containing receptor (cytisine = nicotine > DMPP) (Fig. 3a) and the response was maintained in mutant mice.

The $\beta 2$ -subunit is expressed in the ganglia of wild-type animals⁶⁻⁸, but there was no apparent difference in heart rate or



b Responses to nicotinic agonists

Nucleus	Genotype	No. of cells tested	Responses	Responses (%)
Antero-dorsal thalamus	+/+	22	22	100
	-/-	15	0	0
Latero-dorsal thalamus	+/+	14	14	100
	-/-	9	0	0
Antero-ventral thalamus	+/+	36	36	100
	-/-	10	0	0
Medial habenula	+/+	8	8	100
	-/-	9	8	89

FIG. 3 Patch-clamp recording of nicotine-evoked currents in the MHb and anterior thalamus of $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice. *a*, Representative recordings from cells in the MHb and the anterior thalamus of wild-type and $\beta 2^{-/-}$ mice. The off-rate of the agonist is significantly greater in the MHb than in the anterior thalamus, resulting in a different kinetics of response in the two structures. The response to nicotinic agonists of the MHb is maintained in $\beta 2^{-/-}$ animals, whereas the response to nicotinic agonists of the anterior thalamus is completely abolished in $\beta 2^{-/-}$ mice. *b*, Table of responses to nicotinic agonists in various nuclei of $\beta 2^{+/+}$ and $\beta 2^{-/-}$ mice.

METHODS. Coronal slices were obtained from the thalamus of 8-12-day-old mice using a Dosaka slicer in ice-cold ACSF medium (125 mM NaCl, 26 mM NaHCO_3 , 25 mM glucose, 1.25 mM NaH_2PO_4 , 2.5 mM KCl 2.5, 2 mM CaCl_2 , 1 mM MgCl_2 pH 7.3). Slices were maintained in the same medium for 1-8 h. Cells in slices were visualized through a Zeiss microscope. Whole-cell recordings were obtained with 2-4 M Ω m hard-glass pipettes containing 150 mM CsCl, 10 mM EGTA, 10 mM HEPES, 4 mM di-sodium-ATP, 4 mM MgCl_2 , pH adjusted to 7.3 with KOH. Pulses (5-10 s) of drug were applied rapidly to the cell through a 50 μm diameter pipette above the slice, fed by gravity with a solution containing 150 mM NaCl, 10 mM HEPES, 2.5 mM KCl, 2 mM CaCl_2 , 1 mM MgCl_2 . Recordings were made in the presence of CNQX (5 μM) and of the GABA_A antagonist SR-95531 (10 μM) (R.B.I.). Currents were recorded with an Axopatch 1D (Axon Instrument) patch amplifier, digitized on a Compaq PC and further analysed with the PClamp program (Axon Instrument).

basal body temperature in mutant mice. Spontaneous locomotor activity, which is sensitive to high doses of nicotine and is not modified by drugs selective for the $\beta 2/\alpha 4$ isoform of the nAChR¹⁵, was not significantly different in $\beta 2^{-/-}$, $\beta 2^{+/-}$ and $\beta 2^{+/+}$ mice.

Learning and memory were examined in mutant and wild-type mice using two procedures. The Morris water maze^{16,17} evaluates spatial orientation learning. The performance of $\beta 2^{-/-}$ mice on this test did not differ from that of wild-type mice when tested on the visible platform task (not shown), or on the hidden platform task (minimum swim-time reached after 5 days of training: $\beta 2^{-/-}$ mice ($n=8$), 7.4 ± 1.4 s; wild-type mice ($n=8$), 8.2 ± 2.0 s). In the transfer test both groups of animals spent roughly 35% of the time in the platform quadrant, with the same

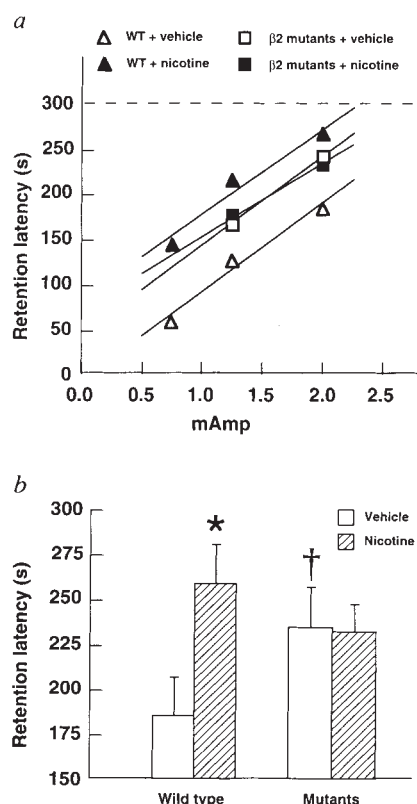


FIG. 4 Performance of $\beta 2^{-/-}$ mice and their wild-type siblings on the passive avoidance test. **a**, Response to various levels of foot shock in retention test following a post-training injection of either vehicle or nicotine ($10 \mu\text{g kg}^{-1}$). Time to first-time entry of the dark chamber (average step-through latency) during the training trial was 17.0 ± 3.6 s for mutant mice and 15.0 ± 3.5 s for their non-mutant siblings. WT, wild-type mice. **b**, Bar graph showing the difference in time spent in the light chamber (retention latency) between wild-type and homozygous $\beta 2$ mutant mice injected with either vehicle or nicotine ($10 \mu\text{g kg}^{-1}$) at foot shock intensity of 2.00 mA. Data are represented as means $\pm$ s.e.m. of the following groups: wild type + vehicle ($n = 27$); wild type + nicotine ($n = 23$); $\beta 2$ -mutant mice + vehicle ($n = 17$); $\beta 2$ -mutant mice + nicotine ($n = 17$). Statistical analysis was performed on the data shown in **a** (obtained at 1.25 and 2.00 mA shock intensity) using a factorial analysis design with 3 between-subject variables and 2 levels for each variable. The analysis indicated a significant interaction between mutant/wild type and vehicle/nicotine factors ($F(1,82) = 5.728, P < 0.05$). The analysis of the interaction of simple effects showed a significant difference between mutant mice and wild-type mice injected with vehicle ($\dagger P < 0.05$), and between vehicle and nicotine treatment of wild-type mice ($* P < 0.01$).

METHODS. Passive avoidance test was as described in the text^{18,19}. In all behavioural tests, each mutant mouse was paired with a control, wild-type animal of the same sex and the same litter (animals were near seven months old for the test). Nicotine bitartrate (Sigma) was freshly dissolved in PBS before each experiment. Intraperitoneal injection of the same volume of either nicotine or vehicle immediately followed foot shock during the training trial.

number of platform crossings ($\beta 2^{-/-}$ mice, 4 ± 0.4 ; wild-type mice, 3.9 ± 0.6).

Retention of an inhibitory avoidance response was assessed using the passive avoidance test, which was also chosen for its pharmacological sensitivity to nicotine administration^{18,19}. This test consisted of a training trial in which the mouse was placed in a well lit chamber of a shuttle box, and the latency to enter the adjacent dark chamber was measured. Upon entry to the dark chamber, a mild, inescapable foot shock was delivered, and vehicle or nicotine ($10 \mu\text{g kg}^{-1}$) was injected into the mouse. After 24 h, retention was assessed by measuring the latency to enter the dark chamber. Time spent in the light chamber (reten-

tion latency) increased proportionally to the applied foot shock in both mutant and wild-type mice. Nicotine alone without a shock has no effect on this test¹⁸. However, treatment with nicotine after foot shock consistently aided retention ($P < 0.01$) by shifting the curve upwards by about 80 s only in wild-type mice (Fig. 4a). Nicotine administration was completely ineffective in $\beta 2^{-/-}$ mice. Interestingly, retention latency was significantly higher for mutant mice than for their non-mutant, vehicle-injected siblings ($P < 0.05$) (Fig. 4b).

Increased retention in the passive avoidance test can be observed in animals with a decreased pain threshold or increased emotionality. Therefore, further behavioural testing was done on all mice included in this experiment. Mutant mice did not differ from their non-mutant siblings for flinch, vocalization or jump response to foot shock (not shown). Possible changes in aversive motivation were studied by measuring exploratory activity in the two-compartment test, which has been pharmacologically validated as an assay of emotionality^{20,21}. In a 15 min test, average time spent and locomotor activity in the dark compartment, as well as transitions between compartments, did not differ between the mutant and wild-type mice (not shown). Although this test does not completely rule out changes in motivation in mutant animals, it is an appropriate control for passive avoidance as both tests use dark/light compartments.

Studies using low doses of nicotine²² or specific nicotine agonists¹⁵ suggest that high-affinity nAChRs in the brain mediate the effects of nicotine on passive avoidance. This study demonstrates that the $\beta 2$ -containing nAChRs make up the high-affinity binding sites for nicotine, and also that nicotine cannot change the performance of $\beta 2^{-/-}$ mice on passive avoidance, providing the first molecular evidence that nAChRs mediate this effect. The enhanced performance of mutant mice versus wild-type mice is quite surprising, however, and further experiments using these mutant mice will help elucidate the mechanism underlying this behavioural difference. These mice provide a model system for studying the pharmacological effects of nicotine in the CNS, and may be useful in elucidating the role of high-affinity nAChRs in cognitive processes, in nicotine addiction and in dementias involving deficits of the nicotinic system. □

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Immunological function of a defined T-cell population tolerized to low-affinity self antigens

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In the thymus there are two major mechanisms of T-lymphocyte tolerance: clonal deletion and clonal inactivation¹⁻³. One important problem underlying the mechanism of clonal inactivation is why unresponsive cells are maintained in the mature peripheral T-cell repertoire. Here we report that transgenic $\alpha\beta$ T-cells may be tolerized to a self antigen Mls-1^a, but still retain proliferative responses for alternative peptide antigens and superantigens. These self-tolerant T cells can also provide immunopathological and memory cytotoxic function *in vivo*. We propose that high-affinity/avidity self-reactive T cells are deleted in the thymus, whereas lower-affinity/avidity interactions lead to unresponsiveness and define the 'resting threshold' for a given T cell. These low-affinity self-tolerant T cells remain functionally competent for high-affinity foreign antigens, and efficiently eliminate natural pathogens *in vivo*.

To examine whether T-cells that have been tolerized by clonal inactivation retain immunocompetence, we have studied a transgenic mouse model that predominantly expresses a V α 2/V β 8.1 T-cell receptor (TCR) specific for the lymphocytic choriomeningitis virus (LCMV)-gp (peptide 33-41) presented by H-2D^b (ref. 4). Because the transgenic receptor uses V β 8.1, potential reactivity to Mls-1^a and staphylococcal enterotoxin B (SEB) exists^{5,6}. Transgenic mice, TCR (H-2^b) were bred with CBA/J (H-2^k, Mls-1^a) animals to generate TCR H-2^{b/k} Mls-1^a transgenic mice. Control transgenic mice were bred with CBA/Ca (H-2^k, Mls-1^b) animals. Analysis of thymocytes using anti-CD4 and anti-CD8 demonstrated that a partial reduction of CD8⁺ thymocytes was apparent in Mls-1^a compared to control Mls-1^b TCR-transgenic mice (Fig. 1a), resulting in a slight decrease of transgenic TCR^{hi} cells (Fig. 1b). A minor reduction of peripheral T cells expressing the transgenic TCR was seen in H-2^{b/k} Mls-1^a mice compared to Mls-1^b controls (Table 1). A decrease in the intensity of the transgenic TCR or CD8 molecules was not seen in Mls-1^a versus Mls-1^b mice (Fig. 1c). No alterations of other cell surface molecules such as LFA-1, CD45, CD44, IL-2R, CD69 or Mel-14 were apparent. Previous studies of this transgenic TCR in H-2^{b/d} Mls-1^a mice have shown that transgenic TCR^{hi} CD8⁺ thymocytes remain undetectable^{4,7}, suggesting that the transgenic receptor has a higher affinity for Mls-1^a presented by I-E^d. Taken together, these profiles suggest that the transgenic receptor can recognize Mls-1^a, resulting in minimal deletion in H-2^{b/k} mice.

To study Mls reactivity in Mls-1^a and Mls-1^b TCR-transgenic animals, CD8⁺ transgenic T cells were enriched by depletion with anti-CD4 and complement. CD8⁺ cells from Mls-1^a H-2^{b/k} TCR-transgenic mice could not respond to Mls-1^a, whereas weak proliferation was detected from CD8⁺ T cells from Mls-1^b H-2^{b/k} mice (Fig. 2a). Spleen cells from TCR RAG2^{-/-} mice (H-2^{b/b}, Mls-1^b) could proliferate to Mls-1^a, thereby excluding the possibility that contaminating CD4⁺ cells or T cells expressing endogenous TCR were responding to Mls-1^a. The TCR RAG2^{-/-} mice did not have mature CD4⁺ cells and CD4⁺ cells were not detected in Mls-1^a-stimulated cultures from CD4-depleted transgenic T cells.

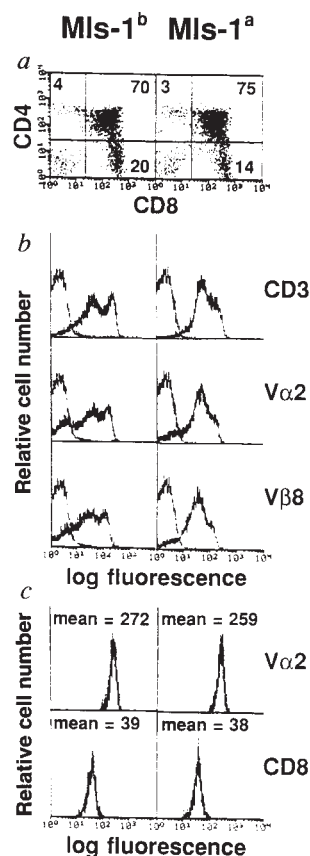


FIG. 1 Partial reduction of CD4⁺CD8⁺ TCR^{hi} thymocytes but no reduction in the intensity of the transgenic TCR or CD8 on peripheral T cells in Mls-1^a TCR-transgenic mice. Thymocytes from H-2^{b/k} Mls-1^b or Mls-1^a TCR-transgenic mice were double-stained with a, PE-anti-CD4 and FITC anti-CD8; or b, single-stained with anti-CD3 (KT3)¹⁸, anti-Va2 (B20.1)¹⁹ or anti-V β 8 (KJ16)²⁰. Dotted lines indicate staining with FITC-goat anti-rat IgG alone. c, Lymph node cells from Mls-1^b or Mls-1^a TCR-transgenic mice were stained with the given antibody in PBS, 2% FCS and 0.1% sodium azide for 30 min at 4 °C. Rat hybridoma supernatants were visualized with FITC-goat anti-rat IgG (TAGO). For double staining using supernatants, residual anti-rat IgG sites were blocked with 2 μ g rat IgG, then incubated with PE-anti-CD8. All PE- or FITC-conjugated monoclonal antibodies were from Pharmingen. Ten thousand viable cells per sample were analysed by FACScan and the Lysis II program (Becton-Dickinson). Representative profiles from at least 5 mice are shown.

METHODS. Single-cell suspension (1×10^6) from the thymuses or lymph nodes were stained with the given antibody in PBS, 2% FCS and 0.1% sodium azide for 30 min at 4 °C. Rat hybridoma supernatants were visualized with FITC-goat anti-rat IgG (TAGO). For double staining using supernatants, residual anti-rat IgG sites were blocked with 2 μ g rat IgG, then incubated with PE-anti-CD8. All PE- or FITC-conjugated monoclonal antibodies were from Pharmingen. Ten thousand viable cells per sample were analysed by FACScan and the Lysis II program (Becton-Dickinson). Representative profiles from at least 5 mice are shown.

Because the transgenic T cells from Mls-1^a mice did not respond to Mls-1^a, we examined whether responsiveness to SEB or the LCMV peptide had been altered. CD4-depleted T cells from TCR-transgenic Mls-1^a and Mls-1^b mice made comparable proliferative responses when co-cultured with varying doses of SEB (Fig. 2b) or LCMV peptide (Fig. 2c) in the absence of exogenous interleukin-2 (IL-2). Crosslinking the TCR using the monoclonal antibody KJ16 also gave similar proliferative responses (Fig. 2d). Comparable results were obtained with TCR RAG2^{-/-} mice (data not shown). Therefore, CD8⁺ T cells that have matured in the presence of Mls-1^a have been selectively tolerized towards the self superantigen Mls-1^a, but can still mount normal proliferative responses to conventional peptide antigens or alternative superantigens.

The functional response of the transgenic T cells was examined *in vivo*. When mice are challenged with LCMV intracerebrally (i.c.), immunocompetent animals succumb to a CD8 T-cell-mediated immunopathological disease within 10 days^{8,9}. Accord-

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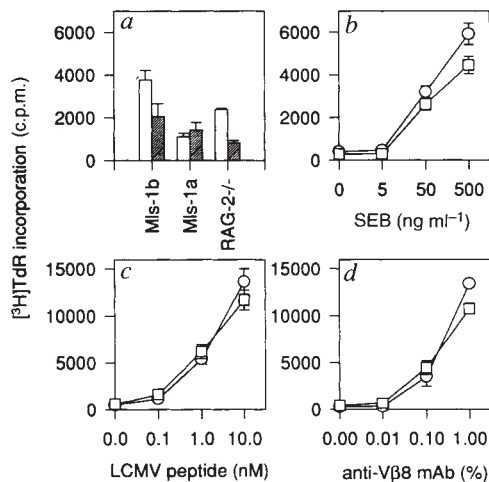


FIG. 2 Proliferative responses of CD8⁺ T cells from Mls-1^a or Mls-1^b TCR-transgenic mice. a, TCR-transgenic CD4-depleted spleen cells (5×10^5) from Mls-1^b or Mls-1^a or TCR-transgenic spleen cells from RAG2^{-/-} mice (1×10^5) were stimulated with 5×10^5 Mls-1^a (white bars) or Mls-1^b (hatched bars) T-cell-depleted spleen cells in the presence of 50 ml^{-1} rIL-2. CD4-depleted spleen cells (1×10^5) from Mls-1^b (○) or Mls-1^a (□) TCR-transgenic mice were analysed for proliferative responses to various concentrations of SEB (b), LCMV peptide-coated macrophages (c), or immobilized anti-Vβ8 (d).

METHODS. Spleen cells from H-2^{b/k} Mls-1^a or Mls-1^b TCR-transgenic mice were treated with RL172.4 and complement. Mls-1^a and Mls-1^b responder cell preparations contained 33% and 36% Vβ8⁺CD8⁺ cells, respectively, and less than 1% CD4⁺ cells. T-cell-depleted spleen cells were prepared from H-2^{b/k} Mls-1^a or Mls-1^b non-transgenic mice by treatment with anti-Thyl (Serotec) and complement and irradiated (1,000 rads). Thioglycollate-induced peritoneal macrophages (H-2^b) were prepared as described²¹ and incubated with various concentrations of LCMV peptide (33–41)²² for 2 h at 37 °C and irradiated. Plates were coated overnight at 4 °C with $10 \mu\text{g ml}^{-1}$ mouse anti-rat IgG (Jackson ImmunoResearch) and incubated with various concentrations of anti-Vβ8 (KJ16) for 2 h at 37 °C. In general, cells were co-cultivated for 3 days, however, in Mls assays, cells were co-cultivated for 4 days and pulsed with ³H-thymidine for the final 14 h. Each data point represents the mean of triplicate cultures $\pm$ s.d. Representative data from at least 3 experiments are shown.

ingly, both Mls-1^a and Mls-1^b TCR-transgenic mice died after LCMV infection (Table 2). Vaccination with LCMV then subsequent i.c. challenge resulted in elimination of the virus and survival of both transgenic Mls-1^a and Mls-1^b mice. Therefore, self-tolerant T cells retained specific effector function in both pathological and memory immune responses *in vivo*.

Previous studies have examined the unresponsive state of T cells bearing a particular β -chain *in vivo*, or T-cell clones *in vitro*^{3,10–13}. The further reactivity of an unresponsive T cell with a defined $\alpha\beta$ pair has not been extensively investigated and functionally analysed *in vivo*. In our model, unresponsiveness to Mls-1^a and reactivity to LCMV and SEB of mature T cells expressing a defined TCR may be related to the affinity/avidity of the T cell for the specific antigen on antigen-presenting cells. The ability of this TCR to recognize Mls-1^a is relatively weak as demonstrated by the partial thymic clonal deletion and reduced proliferation of CD8⁺ T cells (Mls-1^b) to Mls-1^a. Therefore, unresponsiveness to a poorly recognized self molecule is defined and maintained, perhaps because of minor alterations in the levels of adhesion molecules or signalling molecules. The activation of Mls-1^a-tolerant T cells was not overcome by the addition

of exogenous IL-2 or by nonspecific activation through cytokines generated by viral infection¹⁴, because neither reagent or agent was used in the proliferation assays.

Differential responses to TCR/CD3 crosslinking has been previously reported because some anergic T cells do not respond to receptor signalling^{12,15}, whereas in other models a partial or full response^{10,11,16,17} is generated. Collectively, this may reflect the relative affinity/avidity for the ligand that initially induced the unresponsive state as compared to the strength of the TCR crosslinking signal.

One interpretation of our study is that lower affinity/avidity self-reactive interactions may lead to unresponsiveness to self molecules by slightly increasing the 'resting threshold' of the T cell. This permits further activation to higher-affinity cross-reactive antigens and provides an immunological function for self-tolerized unresponsive T cells. It is possible that the T cell may be 'fine-tuned' for different activation thresholds depending upon thymic selection events. □

TABLE 1 Analysis of peripheral T-cell subsets in TCR-transgenic Mls-1^a and Mls-1^b mice

Mice	n	Total lymph node cells (%)			CD8 ⁺ cells (%)	
		CD3	CD4	CD8	Vα2	Vβ8
Mls-1 ^b	6	76 $\pm$ 10	13 $\pm$ 5	58 $\pm$ 10	91 $\pm$ 12	98 $\pm$ 3
Mls-1 ^a	5	72 $\pm$ 7	10 $\pm$ 5	58 $\pm$ 13	79 $\pm$ 9	95 $\pm$ 7

TABLE 2 Functional response of T cells *in vivo*

TCR-transgenic mice	Infection*	Challenge†	Survival	Mean survival (days $\pm$ s.d.)
Mls-1 ^b	—	LCMV i.c.	0/7	5.4 $\pm$ 1.1
	LCMV i.v.	LCMV i.c.	4/4	> 20
Mls-1 ^a	—	LCMV i.c.	0/4	5.0 $\pm$ 0.0
	LCMV i.v.	LCMV i.c.	4/4	> 20

* Mice were infected with 2,000 PFU LCMV-Armstrong intravenously (i.v.) 14 days before i.c. challenge.

† Mice were given an intracerebral challenge (i.c.) with 3×10^4 PFU LCMV-Armstrong.

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An HMG-box-containing T-cell factor required for thymocyte differentiation

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Two candidate genes for controlling thymocyte differentiation, *T-cell factor-1* (*Tcf-1*) and lymphoid enhancer-binding factor (*Lef-1*), encode closely related DNA-binding HMG-box proteins^{1,2}. Their expression pattern is complex and largely overlapping during embryogenesis, yet restricted to lymphocytes postnatally³. Here we generate two independent germline mutations in *Tcf-1* and find that thymocyte development in (otherwise normal) mutant mice is blocked at the transition from the CD8⁺, immature single-positive to the CD4⁺/CD8⁺ double-positive stage. In contrast to wild-type mice, most of the immature single-positive cells in the mutants are not in the cell cycle and the number of immunocompetent T cells in peripheral lymphoid organs is reduced. We conclude that *Tcf-1* controls an essential step in thymocyte differentiation.

Postnatal *Tcf-1* expression in mice is restricted to T lymphocytes³. The onset and kinetics of *Tcf-1* expression within the T-cell lineage was determined by northern blot analysis of thymocyte subpopulations which were separated by fluorescence-activated cell sorting (FACS). Thymocytes were sorted on the basis of the differential surface expression of CD25, CD4, CD44, CD8 and CD3, which define an ordered progression along the T-lineage differentiation pathway^{4,5}. *Tcf-1* expression

peaks in CD8⁺/CD3[−]/CD4[−] T-cell precursors (immature single-positive cells, ISPs) and declines thereafter (Fig. 1a). *Lef-1* messenger RNA is already detectable at the CD25⁺/CD44[−] double-negative (DN) stage and is still present at the double-positive (DP) stage (Fig. 1b).

To determine whether *TCF-1* plays a role in T-cell development after lineage commitment, two independent mutations were introduced into the *Tcf-1* gene by homologous recombination in embryonic stem (ES) cells (Fig. 2). The *Tcf*^{ΔVII} mutation was created by introducing a short deletion into exon VII, which encodes an essential part of the sequence-specific HMG box⁶. The *Tcf*^{ΔV} mutation consisted of a small deletion in exon V, which is conserved between *Tcf/Lef* homologues in *Drosophila* (D. Dooijes and H.C., unpublished), mice and man^{1,7}. In both cases, homologous recombination occurred with high frequency in ES cells (19 and 37% of G418-resistant clones, respectively).

Mice homozygous for either of the two mutations were healthy and fertile and had a normal lifespan. Apart from the lymphoid system, internal organs showed no gross morphological abnormalities. Heterozygous mice had the wild-type phenotype, including a normal T-lymphocyte differentiation pathway (see Fig. 3d for example), arguing against a dominant-negative effect of the mutations.

As shown in Fig. 3a, the thymus of *Tcf*^{ΔVII/ΔVII} mice were greatly reduced in size. Histological analysis revealed an organization into cortex and medulla, but the cortex was narrow and pale-staining, indicative of hypocellularity (Fig. 3b and c). Correspondingly, total thymocyte numbers were reduced 10–100 fold compared to heterozygous or wild-type littermates (Fig. 3d), a difference that became more dramatic with age. *Tcf*^{ΔV/ΔV} thymuses were less-affected, with thymocyte numbers decreased by 2–5-fold (Fig. 3d) at all ages, and no histological aberrations apart from a moderate reduction in size (not shown).

The reduction in thymocyte numbers in both types of mutant mice suggests that disruption of the *Tcf-1* gene interferes with thymocyte development. To define the stage(s) at which this interference occurs, we analysed thymocytes by triple staining for CD3 (or T-cell receptor α/β chains (TCR α/β)), CD4 and CD8 by FACS. In *Tcf*^{ΔVII/ΔVII} thymuses, we found a largely unaffected CD4/CD8 double-negative precursor population. By contrast absolute numbers of DP and mature single-positive (MSP) thymocytes were reduced up to 100-fold compared to littermate controls (Fig. 4a). We consistently observed an

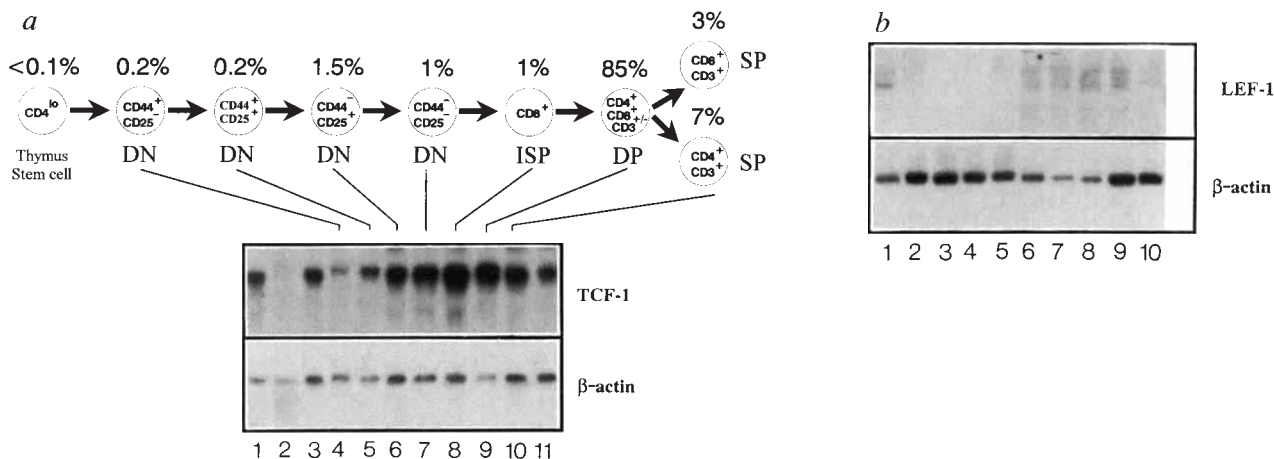


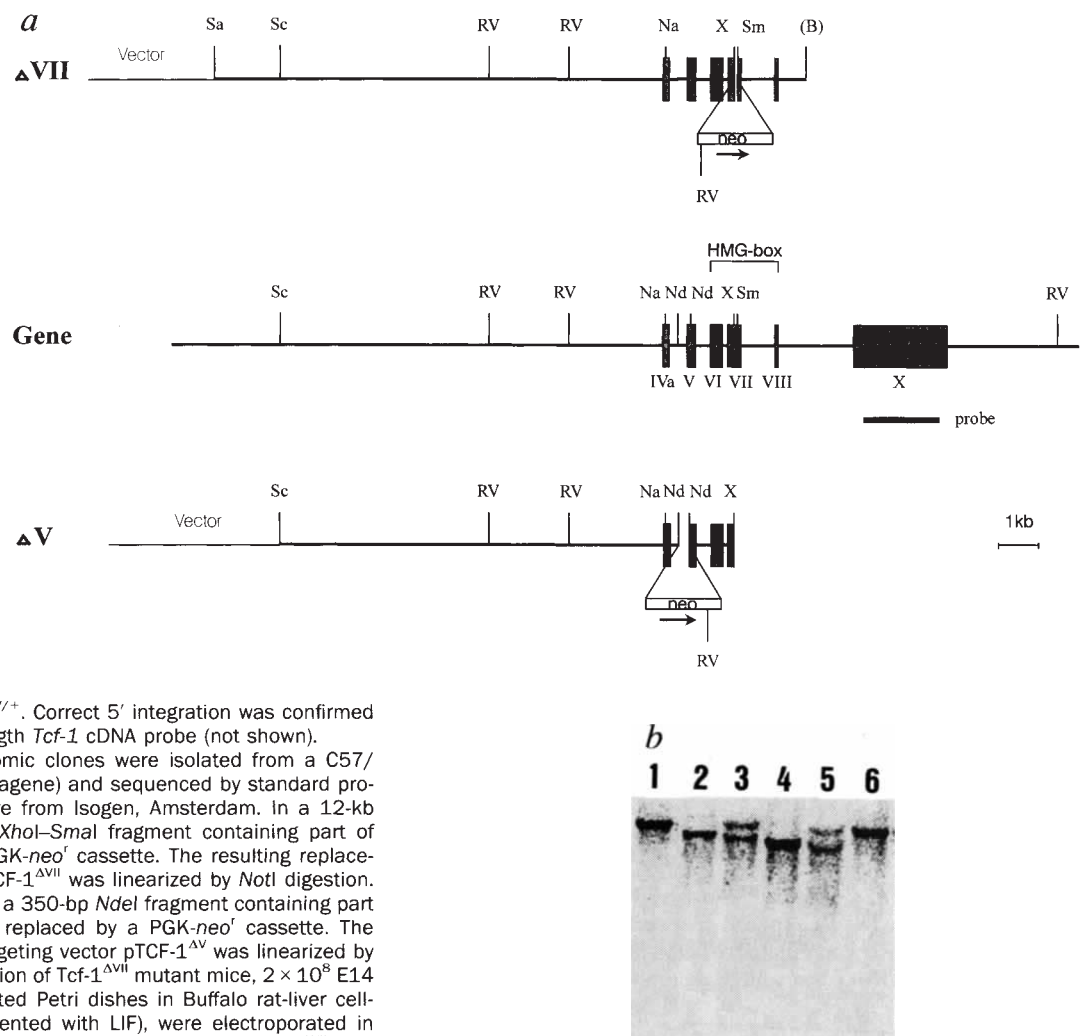
FIG. 1 Stage-specific expression of *Tcf-1* and *Lef-1* mRNA in the T-cell lineage. Surface markers CD44, CD25, CD4, CD8 and TCR/CD3 define an ordered progression in thymocyte differentiation³ (top). Thymocytes were separated on the basis of the expression of these surface markers, as described⁴. Northern blots were probed for *Tcf-1* and for β -actin as a control. *Tcf-1* expression was highest in ISP and DP RNA samples

when normalized for β -actin. *Lef-1* expression, typically appearing as a doublet², was much lower and was detectable from the CD25⁺/CD44[−] to the DP stage. Lanes: 1, TCR α/β cytotoxic T-cell clone; 2, P815 non-lymphoid cell line; 3, TCR γ/δ hybridoma; 4–10, sorted thymocytes of phenotypes indicated above the blot; 11, lymph node T cells.

FIG. 2 Disruption of the murine *Tcf-1* gene and genotypic analysis. **a**, Restriction enzyme maps of *Tcf-1* genomic fragment and the two targeting constructs. Exons are indicated as hatched boxes and numbered according to the human *Tcf-1* gene⁶. Exon IVa and IX represent alternative exons. Restriction sites: RV, *EcoRV*; Sc, *SacII*; X, *XhoI*; Nd, *NdeI*; Na, *NarI*; Sm, *SmaI*; Ns, *NsiI*; B, *BglII*.

b, Southern blot analysis of *EcoRV*-digested genomic DNA from adult progeny of intercrosses of either *TCF-1*^{ΔVII} or *TCF-1*^{ΔV} heterozygotes. A cDNA fragment covering most of exon X was used as a probe. Hybridizing bands of 13, 9 or 10 kb indicate the presence of a *Tcf-1* wild type, *Tcf-1*^{ΔV} or *Tcf-1*^{ΔVII} alleles, respectively. Lanes: 1 and 6, *Tcf-1*^{+/+}; 2, *Tcf-1*^{ΔVII/ΔVII}; 3, *Tcf-1*^{ΔVII/+}; 4, *Tcf-1*^{ΔV/ΔV}; 5, *Tcf-1*^{ΔV/+}. Correct 5' integration was confirmed by hybridization with a full-length *Tcf-1* cDNA probe (not shown).

METHODS. Murine *Tcf-1* genomic clones were isolated from a C57/B6 × C3H EMBL3 library (Stratagene) and sequenced by standard procedures. Oligonucleotides were from Isogen, Amsterdam. In a 12-kb *SacII*–*BglII* fragment, a 67-bp *XhoI*–*SmaI* fragment containing part of exon VII was replaced by a PGK-*neo*^r cassette. The resulting replacement-type targeting vector pTCF-1^{ΔVII} was linearized by *NotI* digestion. In a 9-kb *SacII*–*XhoI* fragment, a 350-bp *NdeI* fragment containing part of intron IV and exon V was replaced by a PGK-*neo*^r cassette. The resulting replacement type targeting vector pTCF-1^{ΔV} was linearized by *XhoI* digestion. For the generation of *Tcf-1*^{ΔVII} mutant mice, 2×10^8 E14 ES cells (grown in gelatin-coated Petri dishes in Buffalo rat-liver cell-conditioned medium supplemented with LIF), were electroporated in 600 μl medium (800 V, 3 μF, 0.1 ms) with 150 μg of the TCF-1^{ΔVII} construct. Targeted cells were selected after one day in 150 μg ml⁻¹ G418. From 127 *neo*^r clones, 24 (19%) had acquired the *neo*^r insertion by homologous recombination. TCF-1^{ΔV} mutant ES cells mice were obtained in a similar fashion. From 310 *neo*^r clones isolated, 106 (37%) had acquired the *neo*^r insertion by homologous recombination. Chim-



aeric animals were generated by injection of heterozygous ES cells into C57B1/6 blastocysts. Germline chimaeras (*F*₀) were obtained from 2 out of 3 injected TCF-1^{ΔV} clones and from 3 out of 5 injected TCF-1^{ΔVII} clones.

increase in the percentage of ISP (CD8⁺/CD3⁺/CD4⁺) thymocytes. Absolute numbers of these precursor cells, which normally represent 0.5–1% of total thymocytes⁸, were increased 3–5-fold in young (3–6-week-old) mutant mice (not shown). In older mice, total numbers of thymocytes were so low that this effect was less clear. Consistent with the expansion of the DN compartment, the proportion of cells staining for the early markers CD25, CD44 and FcγRII/III was markedly increased (not shown). Phenotypic changes in *Tcf*^{ΔV/ΔV} thymocytes were qualitatively similar but less striking. The size of the DN population was normal, whereas the DP and MSP populations were decreased 3–5-fold, compared to littermate controls, thus accounting for the observed decrease in total thymocytes. The ISP compartment was expanded 3–5-fold in absolute numbers at all ages (Fig. 4b).

As expected⁸, ISP cells could be stained with the early marker heat-stable antigen (HSA), although not as much as wild-type ISP cells (Fig. 4c), probably because of their smaller size, as seen in the scatter profiles (not shown). Staining for intracellular TCR-β protein, indicating productive TCR-β rearrangement, was the same in mutant and wild-type ISP cells (not shown). Determination of DNA content revealed that the vigorous cell-cycling of the normal ISP compartment⁸ did not occur in knock-

out ISP cells (Fig. 4c), thereby accounting for the decrease in cell numbers after the ISP stage in mutant mice.

In *Tcf*^{ΔVII/ΔVII} mice, T-cell numbers in lymph nodes and spleen were decreased 10-fold and 3-fold, respectively; the CD4:CD8 and TCRα/β:TCRγ/δ ratios were unaffected. B-cell numbers were normal, as assessed by staining for soluble immunoglobulin and B220, as were natural killer cells, which were detected with the monoclonal antibody NK1.1 (not shown). In *Tcf*^{ΔV/ΔV} mice, there was no significant decrease in T- or B-cell numbers in spleens or lymph nodes (not shown). Splenic T cells from both *Tcf*^{ΔV/ΔV} and *Tcf*^{ΔVII/ΔVII} mice were functional in proliferation assays in response to concanavalin A and alloantigen, as well as in allogeneic cytotoxicity assays (not shown). Furthermore, total IgM and IgG remained the same, as did specific immunoglobulin responses to foreign protein antigens (M. Schilham, unpublished). Thus, despite the pronounced effect of the *Tcf*^{ΔVII} mutation in particular on thymocyte development, mature T lymphocytes did not seem to depend on a functional *Tcf-1* gene product.

The quantitative difference in T-cell numbers correlated with the difference in the amount of mRNA for the two mutant phenotypes. No transcript was detected on a northern blot from the *Tcf*^{ΔVII} allele, but a single, novel mRNA from the *Tcf*^{ΔV}

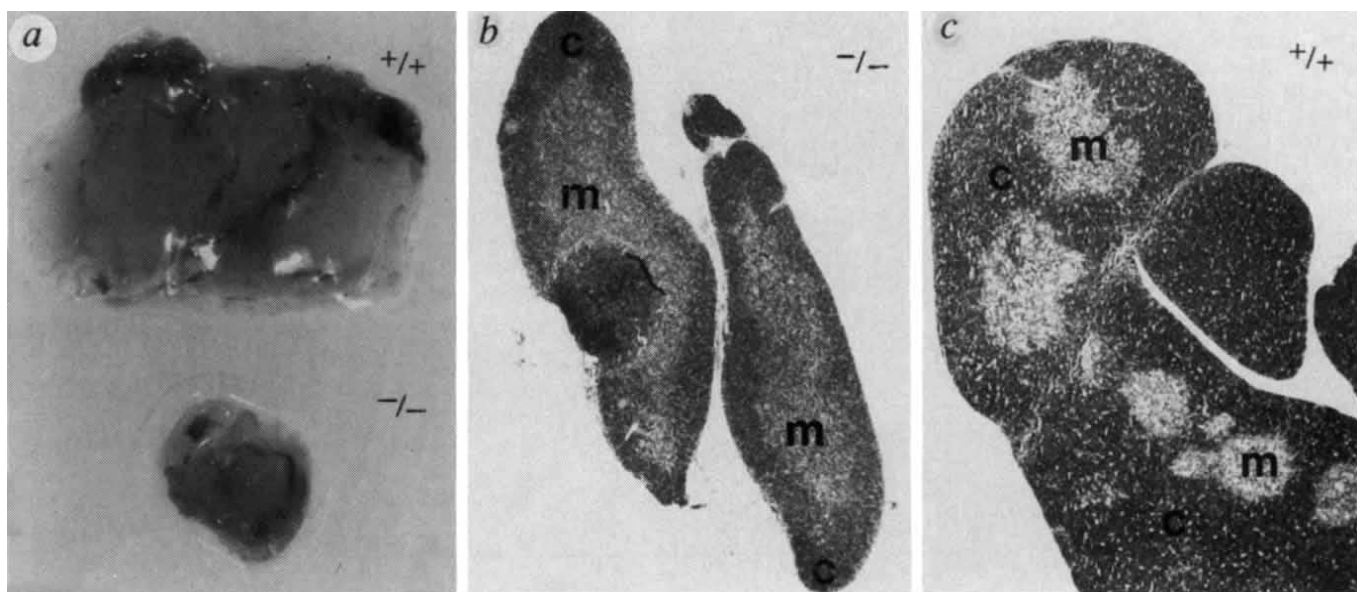


FIG. 3 Thymuses of homozygous $TCF-1^{\Delta VII}$ mice. *a*, Freshly isolated thymuses from 1-month-old knockout ($-/-$), and wild-type littermate ($+/+$). *b* and *c*, Histological analysis of thymuses from *a* at equal magnification. C, Cortex; M, medulla. *b*, Histology of two lobes of the $TCF-1^{\Delta VII/\Delta VII}$ thymus; *c*, histology of 1/3 of one lobe of a $+/+$ thymus. *d*, Total numbers of thymocytes in $TCF-1^{\Delta VII}$ (top) and $TCF-1^{\Delta V}$ (bottom) knockout mice and their littermate controls at the indicated ages.

allele was apparent at $\sim 15\%$ of wild-type level (not shown). In this mRNA, exon IV was spliced to exon VI, resulting in an in-frame deletion. The predicted truncated protein retains the sequence-specific HMG box and presumably at least part of its biological activity.

So far, no disruptions in transcription factor genes have been reported that uniquely affect the T-cell lineage. The T lymphocyte has been the focus of many knockout experiments: genes encoding TCR α - and β -chains^{9,10}, *Lck* tyrosine kinase¹¹, *Rag* recombinases that mediate rearrangement of TCR and immunoglobulin genes^{12,13}, and accessory surface molecules such as CD4 and CD8 α ^{14,15}, have all been disrupted. The resulting phenotypes can be divided into (1) 'early' phenotypes, in which the total number of thymocytes is severely decreased as a result of a block at the DN stage, and (2) 'late' phenotypes, in which numbers of thymocytes are normal because the block is at the DP stage or later. *Tcr- β* , *Rag* and *Lck* gene disruption results in typical early phenotypes^{9,11,13}; *Tcr- α* , CD4 and CD8 α disruption cause a typical late phenotype^{9,10,14,15}. The *Tcf-1* knockout phenotype should be classified as an early phenotype, but is unique in that the block occurs at the ISP-to-DP transition and not in the DN stage. Therefore *Tcf-1* cannot be positioned upstream of the other 'early' genes *Lck*, *Tcr- β* or *Rag-1* and -2. Similarly, no simple hierarchical relationship can exist between *Tcf-1* and *Tcr- α* or other late genes, as *Tcf-1* gene disruption would then give a late phenotype.

The molecular events controlled by *Tcf-1* are unknown. *Tcf-1* does not behave as a typical transcriptional activator, as measured in cotransfection CAT assays¹⁶. It contains a DNA-binding HMG box with specificity for the A/T A/TCAAAG-motif, which is present in many T-cell enhancers^{17,18}. Sequence-specific HMG-box factors may act as architectural elements to organize the spatial structure of enhancers²⁰. *Lef-1* shows identical sequence preference² and, given its expression in early thymocytes (Fig. 1*b*), might be responsible for the 'leakiness' of the *Tcf-1* mutation. *Lef-1* gene disruption does not affect the T lineage, but

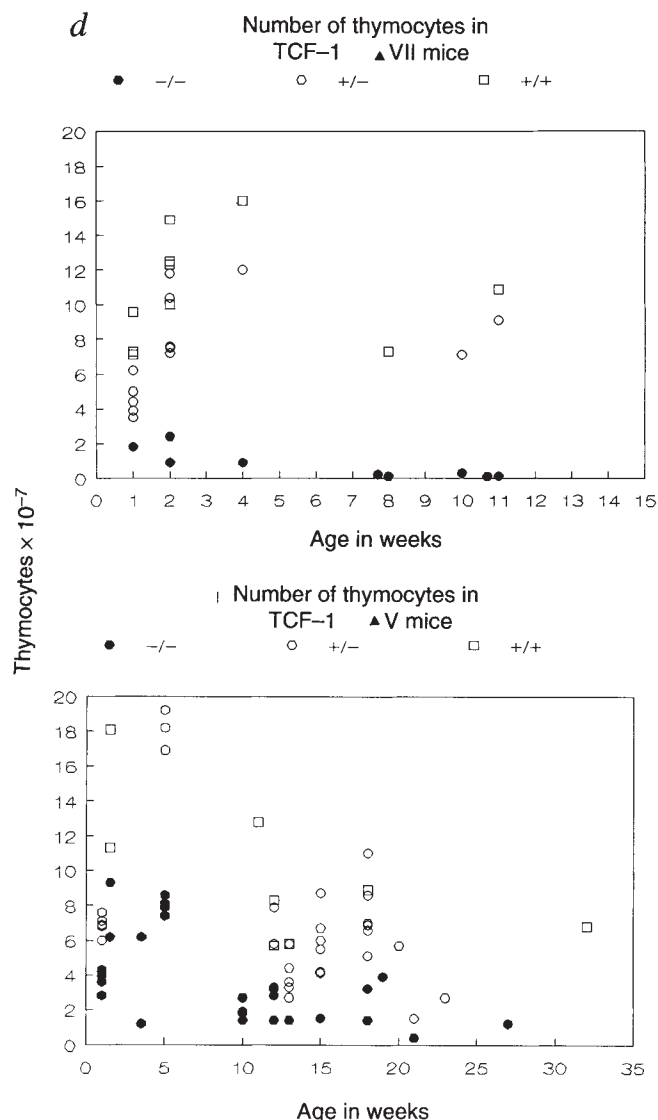
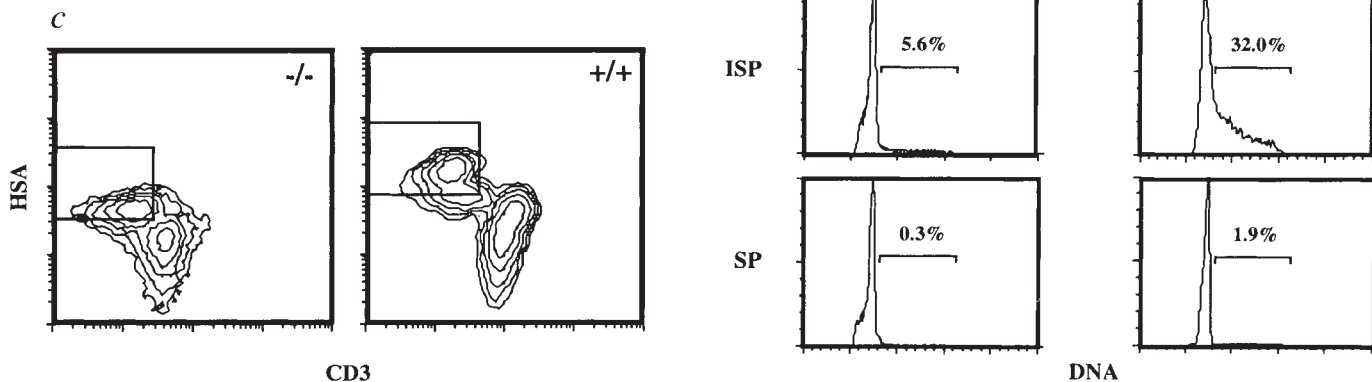
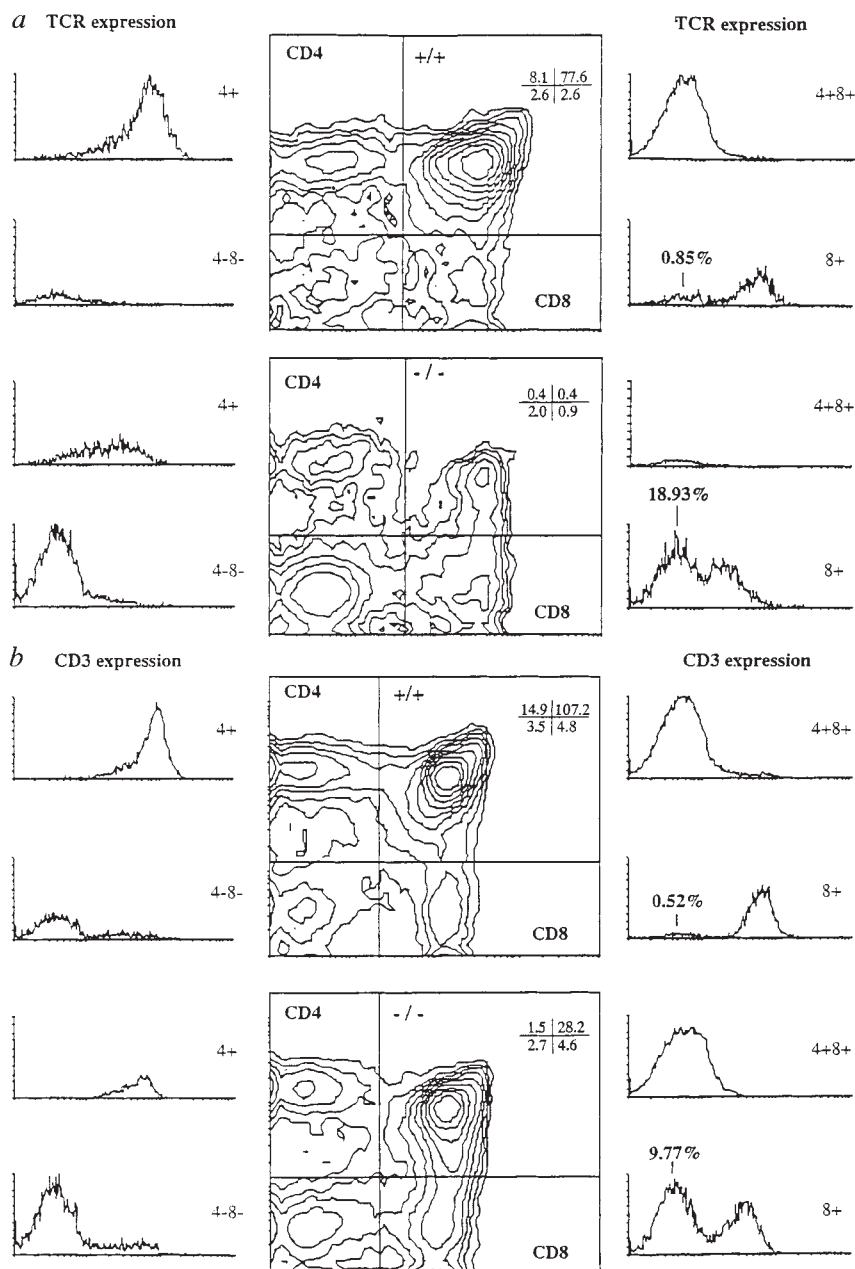


FIG. 4 Phenotypic and cell-cycle analysis of *Tcf-1* knockout thymocytes. *a*, Triple TCR/CD4/CD8 staining of *Tcf-1*^{ΔV/ΔV} thymocytes (bottom) compared to a littermate control (top). Absolute numbers of cells in CD4/CD8 quadrants are given at the top right of each panel. Gates set to analyse TCR expression coincided with the cross set for statistical analysis. TCR or CD3 expression in arbitrary units is given directly adjacent to the pertinent CD4/CD8 quadrant. Percentage of ISP cells are indicated (0.85 and 18.93%, respectively). *b*, As *a*, but for *Tcf-1*^{ΔV/ΔV} thymocytes (bottom). *c*, HSA/CD3 surface expression (top) and cell-cycle analysis (bottom) of CD8 single-positive cells in *Tcf-1*^{ΔV/ΔV} (left) and wild-type thymocytes (right). HSA^{high}/CD3⁺ are typical ISP cells⁸. HSA^{low}/CD3⁺ cells represent mature single-positive thymocytes. ISP cells in the indicated gates were sorted and their DNA content determined. 32.0% of control versus 5.6% of mutant ISP cells were in S or G2/M phase. Although mature CD8⁺ cells (SP; sorting gate not indicated) were mostly non-cycling, differences were again observed between control and mutant mice.

METHODS. Four-colour staining and FACS sorting was done using the antibody conjugates anti-CD8 Red⁶¹³ (Gibco BRL), anti-CD4 allophycocyanin (Caltag), anti-CD3 FITC (purified and conjugated by A.W. from clone 17A2, and anti-CD24 (HSA) phycoerythrin (Pharmingen). Live gating and sorting on a FACStar⁺ (Becton Dickinson) was done at three levels: for viable cells on FSC and SSC, for CD4⁺CD8⁺ cells based on FL3 and FL4 and then for the indicated populations of CD24⁺CD3^{+/lo} (that is, ISP) and CD24^{-/-lo}CD3⁺ (that is, SP) based on FL1 and FL2. Sorted cells were analysed for DNA content after addition of 250 μg ml⁻¹ propidium iodide in 1% NP-40. Percentage S and G2/M cells was determined using the Double Discrimination Module on the FACScan. All data were analysed using the Lysys program (Becton Dickinson).



has striking effects on tooth, hair follicle and mammary gland development and on the trigeminal nucleus in the brain¹⁹.

Members of the sequence-specific HMG-box family, could turn out to be important developmental regulators. The *Tcf-1* knockout mouse represents an accessible model system for HMG-box-factor-mediated developmental control in mammals. □

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Conversion of antagonist-binding site to metal-ion site in the tachykinin NK-1 receptor

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MUTATIONAL analysis of the tachykinin NK-1 (refs 1–7), NK-2 (ref. 8) and angiotensin AT-1 (refs 9, 10) receptors indicates that non-peptide antagonists act through residues located between the seven transmembrane segments, whereas natural peptide agonists bind mainly to residues scattered in the exterior part of the receptor^{1–4,7,11–13}. The presumed contact points for the prototype NK-1 antagonist CP96,345 cluster on opposing faces of the outer portions of transmembrane helices V and VI (refs 1–5). Here we show that systematic introduction of histidyl residues at this antagonist-binding site in the human NK-1 receptor gradually converts it into a high-affinity metal-ion-binding site without affecting agonist

binding. In a double mutant with histidine residues substituted at the top of transmembrane segments V and VI, respectively, Zn²⁺ inhibits binding of radiolabelled agonist peptide and efficiently blocks phosphoinositol turnover induced by substance P. We propose that Zn²⁺ and CP96,345 act as 'allosteric competitive' antagonists by stabilizing inactive conformations of the mutant and the wild-type receptor respectively. Introduction of metal-ion-binding sites could be used as a general tool in the structural and functional characterization of helix-helix interactions in G-protein-coupled receptors, as well as in other membrane proteins.

On the basis of the apparent spatial proximity of the putative contact points for CP96,345 in the molecular model of the human NK-1 receptor (Fig. 1), we attempted to exchange the binding site for the non-peptide antagonist with a metal-ion-binding site in order to test this model and the mechanism of action of the non-peptide antagonist^{11,14} by using a considerably smaller and structurally unrelated metal-ion. The structures of several naturally occurring metal ion sites are known^{15–17} and similar sites have been built into other proteins and into model proteins^{18–20}. Histidine residues were incorporated at selected positions in and around the putative binding site for CP96,345 and the ability of the non-peptide antagonist and Zn²⁺ to inhibit the binding of radiolabelled substance P (the natural peptide agonist) was monitored. One of the suggested interaction points in transmembrane segment V (TM-V) is already a histidine, namely His 197 (HisV:05)². Substitution of Glu 193 (GluV:01), one helical turn above this point, with a histidine increased the

TABLE 1 Binding of peptide agonist, non-peptide antagonist and metal ions to wild-type and histidine-substituted NK-1 receptor mutants

	Helical segment	Substance P				CP96,345				ZnCl ₂				CuCl ₂			
		K _d (nM)	±s.e.m.	(n)	F _{mut}	K _i (nM)	±s.e.m.	(n)	Fold decrease	K _i (μM)	±s.e.m.	(n)	Fold increase	K _i (μM)	±s.e.m.	(n)	Fold increase
hNK-1		0.21	±0.02	(13)	1.0	0.25	±0.02	(10)	1	492	±42	(7)	1	346	±49	(6)	1
[E193H]	TM-V	0.12	±0.01	(8)	0.6	1.54	±0.09	(5)	6	11	±2	(5)	44	116	±24	(4)	3
[T201H]	TM-V	0.23	±0.05	(4)	1.1	14.2	±3.2	(3)	57	50	±0	(3)	10	49	±23	(2)	7
[F268H]	TM-VI	0.52	±0.08	(6)	2.5	1.17	±0.19	(3)	5	38	±2	(5)	13	42	±20	(2)	8
[L269H]	TM-VI	0.11	±0.01	(7)	0.5	0.41	±0.03	(5)	2	73	±3	(5)	7	167	±18	(4)	2
[Y272H]	TM-VI	0.05	±0.01	(9)	0.2	0.50	±0.05	(5)	2	5	±1	(5)	94	393	±49	(5)	1
[F268H;Y272H]	TM-VI	0.74	±0.31	(3)	3.5	4.00	±0.97	(4)	16	4	±1	(4)	123	313	±32	(2)	1
[L269H;Y272H]	TM-VI	0.05	±0.01	(5)	0.2	6.12	±1.23	(3)	24	17	±0	(3)	29	329	±60	(2)	1
[E193H;F268H]	TM-V and -VI	1.41	±0.46	(5)	6.7	6.00	±1.45	(3)	24	19	±1	(5)	26	133	±79	(2)	3
[E193H;L269H]	TM-V and -VI	0.15	±0.02	(7)	0.7	5.63	±0.65	(4)	23	12	±2	(5)	40	63	±15	(4)	5
[E193H;Y272H]	TM-V and -VI	0.34	±0.04	(14)	1.6	34.3	±5.8	(8)	137	0.62	±0.10	(7)	794	37	±10	(6)	9
[H197A]	TM-V	0.12	±0.02	(6)	0.6	33.2	±2.7	(4)	133	1,517	±268	(5)	0.3	399	±55	(6)	1
[H265A]	TM-VI	0.10	±0.02	(6)	0.5	0.29	±0.04	(4)	1	112	±7	(6)	4	185	±40	(6)	2

Gradual conversion of the putative binding site for the non-peptide antagonist CP96,345 to a Zn²⁺-binding site by substitution with His residues. Construction of receptor mutants: residues in the human (h) NK-1 receptor were selected for His substitution on the basis of previous mutational analysis of the CP96,345-binding site^{1–5} plus neighbouring residues Thr 201 and Leu 269, and on the structure of natural and artificial metal-ion sites^{16,18}. Mutants were constructed using oligonucleotide-directed mutagenesis and recombinant polymerase chain reaction (PCR)²⁸. cDNAs encoding wild-type and mutant receptors were cloned into a pTEJ-8 expression vector²⁹, all mutations were verified by restriction endonuclease mapping and DNA sequence analysis. Cloned receptors were transiently expressed in COS-7 cells transfected by calcium phosphate precipitation 2 days before the binding assay³⁰. Binding was assayed using whole cells at 4 °C and 40 pM mono-iodinated [¹²⁵I]-Bolton Hunter substance P (¹²⁵I-BH-SP) as described³⁰. Determinations were made in triplicate (SP, CP96,345) or duplicate (ZnCl₂, CuCl₂). Binding data were analysed and IC₅₀ values determined by nonlinear regression analysis using the Inplot 4.0 software (GraphPad Software, San Diego). Values of the dissociation and inhibition constants (K_d and K_i) were estimated from competition binding using the equations K_d = IC₅₀ - L and K_i = IC₅₀ / (1 + L/K_d), where L is the concentration of radioligand.

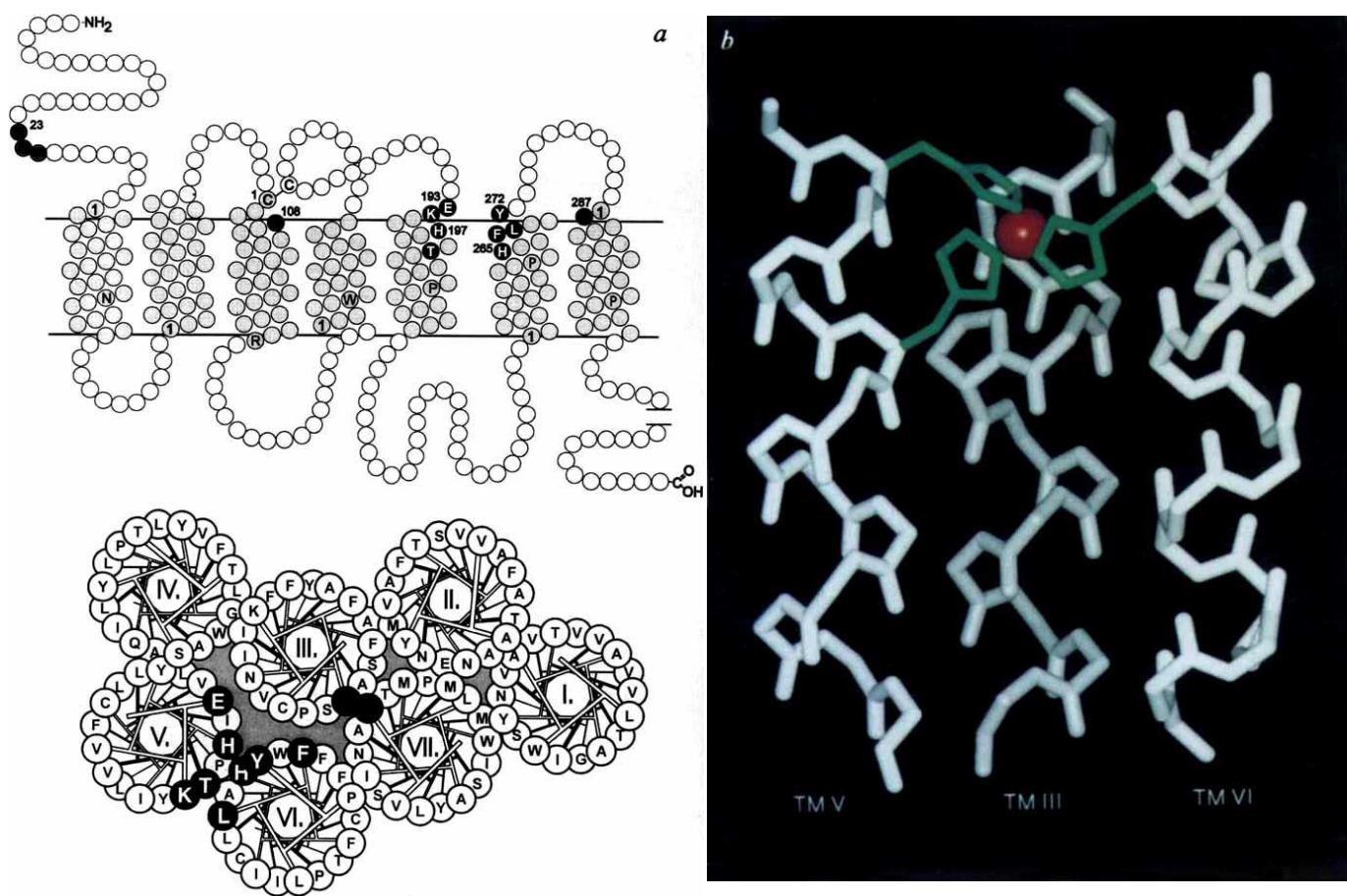


FIG. 1 Diagrams of the human NK-1 receptor with the putative binding site for the non-peptide antagonist CP96,345 and a hypothetical molecular model of the introduced Zn^{2+} -binding site at the top of transmembrane segments V and VI. *a*, Serpentine and helical-wheel diagram of the NK-1 receptor, with the putative binding site for CP96,345 as previously identified by mutational analysis¹⁻⁵ indicated in white on black symbols. Two neighbouring residues, Thr 201 and Leu 269, which were also probed by His substitution but which are not directly involved in CP96,345 binding, are also indicated in white on black symbols. Residues presumed to be part of the substance P agonist-binding site are indicated in black on grey symbols⁷. Some residues in TM-II have also been implicated in substance-P binding^{7,13}, but only indirectly, as

they affect only the ability of the agonist to compete for binding with radiolabelled antagonist and not the high-affinity agonist binding itself¹¹. The highly conserved fingerprint residues in each transmembrane helix are also indicated¹². An anticlockwise orientation of the helical wheels is shown in an outside-inwards view of the receptor. The model is built over the bovine rhodopsin structure²⁴ according to refs 12 and 25. *b*, Molecular model of the hypothetical coordination of a Zn^{2+} ion in the [E193H;Y272H]NK-1 receptor construct. Backbones of the α -helices of TM-III, -V and -VI are shown in white, with the presumed participant histidine side chains at positions 193, 197 and 272 (green) chelating a single zinc ion (red).

apparent affinity for Zn^{2+} 44-fold and decreased the affinity for CP96,345 6-fold (Fig. 2), whereas His substitution of Thr 201 (ThrV:09) one helical turn below His 197 increased the affinity for Zn^{2+} 10-fold (Table 1). Substitution in TM-VI of another CP96,345 interaction point, Tyr 272 (TyrVI:24)⁵, increased the affinity for Zn^{2+} 94-fold (Fig. 2), whereas substitutions of a third interaction point, Phe 268 (PheVI:20), or the neighbouring Leu 269 (LeuVI:21) one helical turn below Tyr 272 only increased the affinity for Zn^{2+} by 13- and 7-fold, respectively. Combining the histidine substitutions at position 272 (in TM-VI) or at position 193 (in TM-V) with those at positions 268 or 269 (in TM-VI) did not further increase the ability of Zn^{2+} to compete for substance-P binding to the NK-1 receptor (Table 1). But the double mutant E193H;Y272H (single-letter amino-acid code) had a 794-fold increased affinity for Zn^{2+} and a 137-fold decreased affinity for CP96,345 compared with the wild-type human NK-1 receptor, whereas the affinity for substance P was virtually unchanged (Fig. 2 and Table 1). In saturation binding experiments using radiolabelled substance P in the [E193H;Y272H]NK-1 construct, Zn^{2+} appeared to act as a competitive antagonist, decreasing the dissociation constant K_d in a dose-dependent manner with only a minimal effect on the B_{max}

(number of binding sites) (Fig. 2c). The precise stoichiometry of zinc ions bound to imidazole side chains cannot be determined, but we believe that His 197, which is located at an optimal $i+4$ position^{15,16} in relation to His 193 in helix V, participates in the high-affinity binding of a single Zn^{2+} ion together with the two introduced His residues. This is supported by the negative effect of alanine substituting for His 197 on zinc-ion binding in the wild-type receptor (Table 1). Also, high-affinity binding of Zn^{2+} is achieved in the κ opiate receptor by substituting histidines at these three positions (K. Thirstrup, C. E. E., S. A. Hjorth, T. W. S., unpublished results). In none of the constructs does the apparent affinity for Cu^{2+} increase more than 9-fold, indicating that a relatively specific zinc-binding site has been created (Table 1).

In the wild-type NK-1 receptor, CP96,345 inhibits the substance-P-induced increase in inositol phosphate turnover at a half-maximal effective concentration (EC_{50}) of 18 nM which, in agreement with the substitutions in the presumed binding site for CP96,345, decreases to 196 nM in the [E193H;Y272H]hNK-1 construct. In contrast, Zn^{2+} acts as an antagonist on the mutant NK-1 receptor blocking substance-P-induced inositol phosphate turnover with an EC_{50} of 18 μM (Fig. 3). Thus, exchanging two

residues for histidines at the top of TM-V and -VI in the presumed binding site for the non-peptide antagonist CP96,345 converts this site into a metal-ion-binding site, and Zn^{2+} becomes an antagonist on the NK-1 receptor.

The different binding of CP96,345 and substance P to the NK-1 receptor was at first unexpected¹, but systematic mutational analysis of the NK-1 receptor indicated that these two competitive ligands do not share any points of strong interaction on the receptor²⁻⁵. Moreover, a series of chemically distinct non-peptide antagonists all act independently of the natural agonist by binding to TM-VI, in some cases with additional interaction points on TM-V and in others with residues in TM-VII (ref. 12). As an alternative to the proposed 'volume-exclusion effect'¹³, we have suggested that an 'allosteric competitive' mechanism may operate, in which non-peptide antagonists and peptide agonist compete for the receptor as such by mutually exclusive binding to different sites occurring in distinct conformations of the

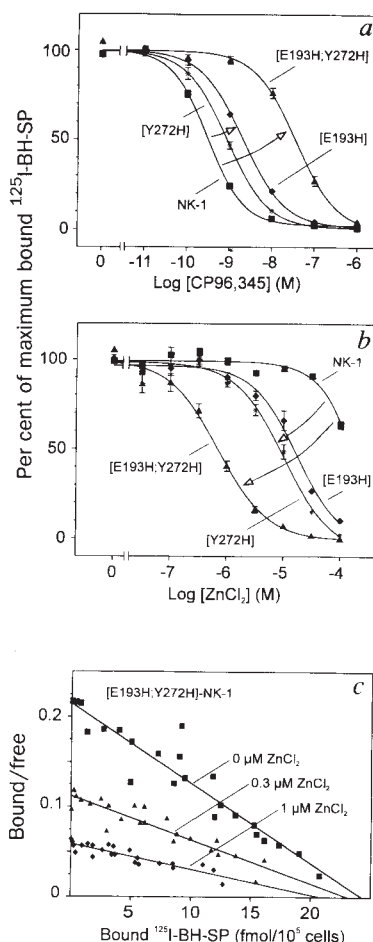


FIG. 2 Loss of binding of non-peptide antagonist CP96,345, and gain of Zn^{2+} binding following introduction of His residues at positions 193 and 272 in the human NK-1 receptor. Competition binding with a, CP96,345, and b, ZnCl_2 in wild-type human NK-1 (■), [E193H]NK-1 (◆), [Y272H]NK-1 (*) and [E193H;Y272H]NK-1 (▲) receptors using ^{125}I -BH-substance P and performed in COS-7 cells as described in Table 1 and ref. 30. Data are expressed as mean $\pm$ s.e.m.; $n = 7-10$. c, Scatchard plots of saturation binding data using increasing concentrations of ^{125}I -BH-substance P in [E193H;Y272H]NK-1 expressed transiently in COS-7 cells in the absence (■) and in the presence of 0.32 (▲) and 1.0 (◆) μM ZnCl_2 and the same conditions as for competition binding. K_d was 0.20 (without ZnCl_2), 0.36 (0.32) and 0.61 nM (1.0 μM ZnCl_2); B_{max} was 24 (without ZnCl_2), 23 (0.32), and 21 fmol per 10^5 cells (1.0 μM ZnCl_2).

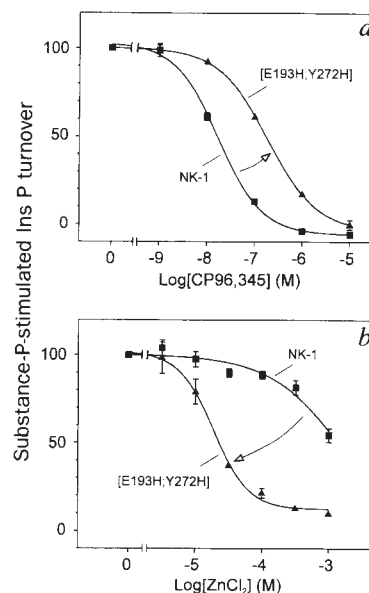


FIG. 3 Loss of antagonist function of the non-peptide CP96,345 and gain of antagonist function of Zn^{2+} following introduction of His residues at positions 193 and 272 in the human NK-1 receptor. Transfected COS-7 cells were incubated for 24 h with 5 μCi of [^3H]-myo-inositol (Amersham) in 1 ml inositol-free Dulbecco's 1885 medium supplemented with 10% fetal calf serum and 2 mM glutamine. Inositol phosphate turnover was stimulated by 1 nM of substance P and antagonized with increasing concentrations of a, CP96,345, or b, ZnCl_2 . Cells were extracted with perchloric acid, and [^3H]inositol phosphates purified on Bio-Rad AG 1-X8 anion-exchange resin as described³. Data are expressed as mean $\pm$ s.e.m.; $n = 3$. EC_{50} values for CP96,345: NK-1, 18 ± 1 nM; [E193H;Y272H]NK-1, 196 ± 9 nM. EC_{50} values for ZnCl_2 : NK-1, >1 mM; [E193H;Y272H]NK-1, 18 ± 1 μM .

receptor¹¹⁻¹⁴. Many antagonists for monoamine transmitters may also function in an agonist-independent way, as negative antagonists that inhibit the constitutive activity of the receptor²¹⁻²³. Our results show that the high-affinity coordination of a small metal ion by residues that normally bind the non-peptide agonist can block peptide binding and function. We propose that the action of the metal ion on the mutant receptor mimics that of CP96,345 on the wild-type receptor and that both act as allosteric competitors, stabilizing a receptor conformation that cannot bind peptide agonist.

The first structures of G-protein-coupled membrane proteins are now being characterized by cryo-electron microscopy²⁴. Although models can be built from these low-resolution structures, the allocation of electron density to different helices is uncertain in the projection map²⁵, as is the overall orientation of the seven-helical bundle^{12,25}. Preliminary attempts have been made to investigate the helix-helix interaction in seven-helix transmembrane receptors by mutagenesis^{26,27}. Artificially created metal-ion-binding sites offer another way to probe helix-helix interactions in these and other membrane proteins, an approach favoured by the relatively small size of the metal ion and the well known geometry of its coordination by amino-acid side chains.

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Editing of glutamate receptor subunit B pre-mRNA *in vitro* by site-specific deamination of adenosine

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EDITING of the glutamate receptor subunit B (GluR-B) pre-mRNA at a single adenosine residue results in an amino-acid change that profoundly alters the electrophysiological properties of the receptor^{1–7}. Here we show that the GluR-B pre-mRNA is efficiently and accurately edited *in vitro*, and that base-pair interactions between the editing site and a sequence in the downstream intron⁸ are required for substrate recognition. In addition, we directly demonstrate that editing results from the conversion of adenosine to inosine by enzymatic deamination. The biochemical properties of this GluR-B editing activity are similar to those of a double-stranded-RNA-dependent adenosine deaminase^{9–15}, but RNA competition and column fractionation experiments indicate that the GluR-B editing and deaminase activities are distinct. Thus, the GluR-B editing enzyme may contain the adenosine deaminase, or a similar activity, and an RNA recognition subunit that specifically targets the enzyme to the editing site.

GluR-B RNA synthesized *in vitro* was accurately and efficiently edited *in vitro* (Fig. 1). The RNA was incubated in nuclear extracts from HeLa or neuroblastoma (N18 TG2) cells, and RNA editing was assayed by reverse transcription of the RNA, followed by polymerase chain reaction (PCR). Amplified

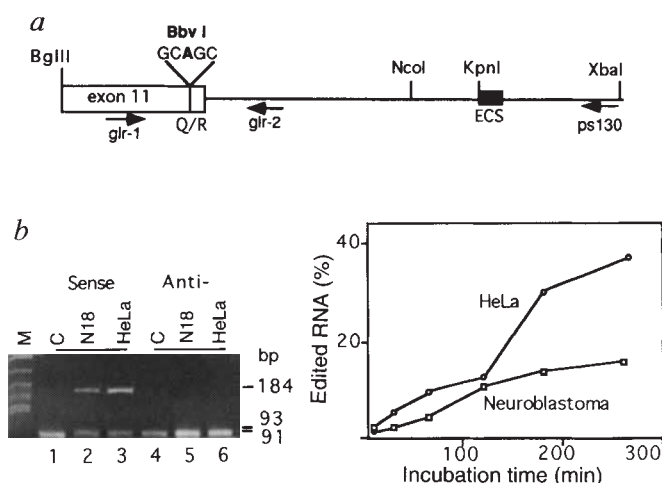


FIG. 1 Reverse-transcription PCR assay of GluR-B RNA editing. *a*, Schematic diagram of the GluR-B construct used in the *in vitro* and *in vivo* editing reactions. A 590-nucleotide fragment of the GluR-B gene was subcloned into pcDNA1/amp beginning at a BglIII site 188 nucleotides upstream of the edited nucleotide and extending through the exon complementary sequence (ECS)⁸ to a HindIII site. *b*, RT-PCR analysis of the editing products. Sense and antisense RNA were synthesized and incubated with no extract (C for controls, lanes 1, 4), nuclear extracts from neuroblastoma N18 TG2 cells (lanes 2, 5), or HeLa cells (lanes 3, 6). After incubation, GluR-B RNA was subjected to RT-PCR and the resulting DNA products (184 bp) were digested with BbvI, producing fragments of 91 and 93 bp when no editing occurred. The BbvI-resistant DNA was cloned, and 14 of the 24 clones contained a guanosine at the editing site. Three of the clones had an additional base change at a site 60 bp downstream of the editing site, which was also found *in vivo*⁸. The remaining clones were unedited and were probably due to incomplete digestion of the amplified DNA by BbvI. Lane M contains λ DNA digested with BstEII. The time course of *in vitro* RNA editing with HeLa or neuroblastoma nuclear extracts is shown on the right.

METHODS. *In vitro* editing was carried out under splicing conditions with modifications¹⁹, typically in 10 μ l, with 50% nuclear extract in editing buffer (20 mM HEPES, pH 7.9, 10 mM EDTA, 40 U ml⁻¹ RNasin) and about 10 fmol RNA substrate, at 25 °C for 4–6 h. This was followed by treatment with proteinase K, reverse transcription (ps130: CGTCTA-GAGTTGACCTGTAGGAAAAATCTAACCTC) and PCR (glr-1: GTCGAGAAACACAAAGTAGTGAATCA, and glr-2: CACCAGGGCTTTATAGTGAATTC-ATAGACA), and analysis on a 3% agarose gel.

double-stranded DNA derived from the unedited RNA is cleaved by the restriction enzyme BbvI at the editing site (GCAGC). Editing, however, alters this site, generating a BbvI-resistant sequence (GCGGC)¹⁶. After incubation with extract, increasing amounts of edited RNA accumulated, as reflected by the generation of amplified DNA resistant to BbvI (Fig. 1b). No BbvI-resistant DNA was observed when control antisense GluR-B RNA was incubated with extract. Sequence analysis of the cloned PCR products confirmed that the BbvI-resistant DNA was accurately edited. When the same GluR-B RNA was expressed in neuroblastoma cells *in vivo*, about 40% of the RNA was edited (data not shown).

The chemical nature of the modification of the adenosine at the editing site has not been previously determined. An adenosine is present at the editing site in genomic DNA, whereas a guanosine is present at this site in complementary DNA copies of the messenger RNA. Therefore, editing is likely to result from deamination of adenosine in RNA to inosine and subsequent incorporation of guanosine at this site during cDNA synthesis. We examined the nature of the chemical modification of adenosine directly by preparing GluR-B RNA in which the adenosine at the editing site was specifically labelled (Fig. 2a)¹⁷. After *in vitro* editing, the RNA was digested with nuclease P1 to produce 5'-monophosphate nucleotides. Thin-layer chromatographic

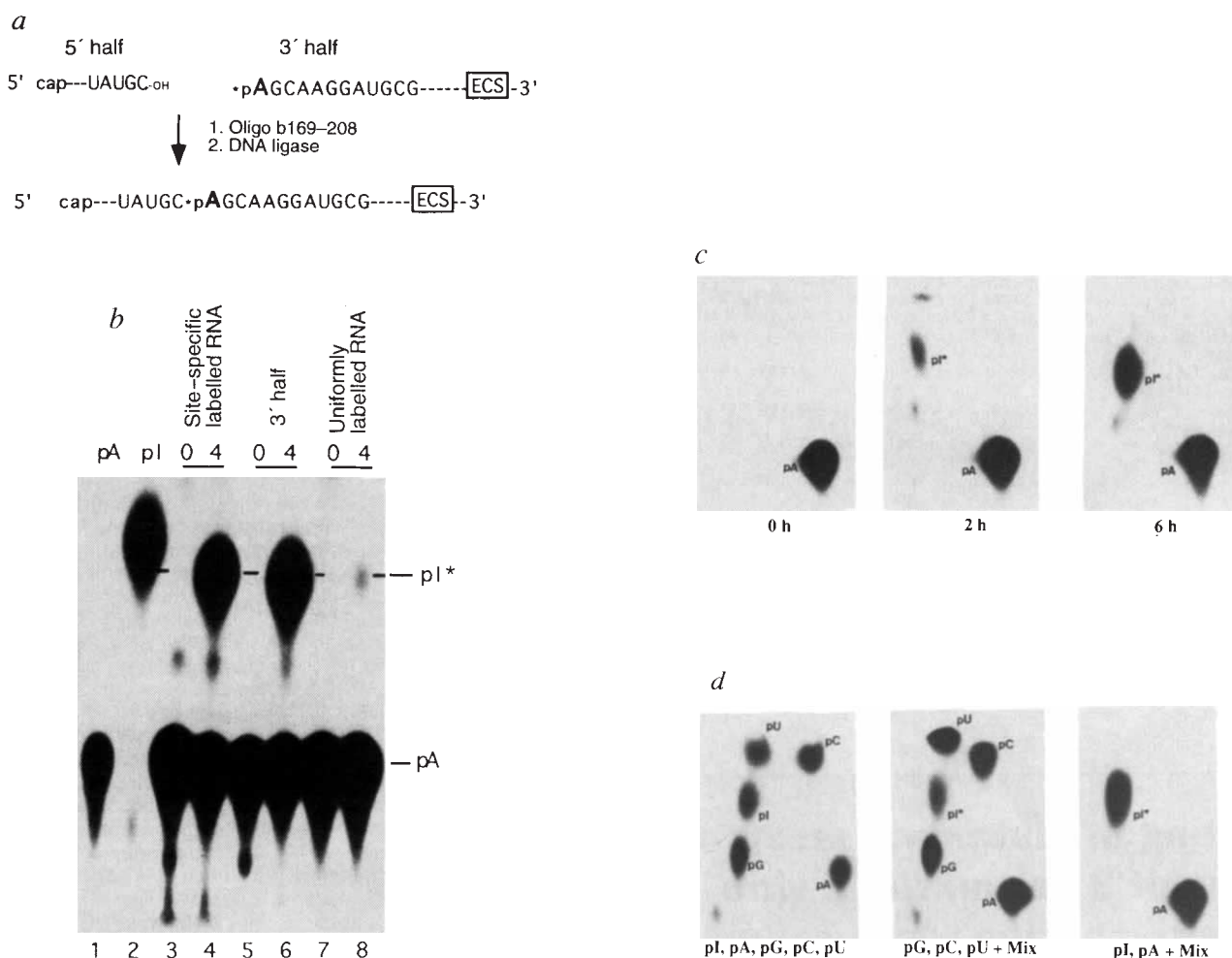


FIG. 2 Thin-layer chromatography (TLC) analysis of GluR-B RNA editing. **a**, The 5'-half GluR-B RNA (185 nucleotides (nt) upstream of the editing site) and 3'-half GluR-B RNA (408 nt downstream of the editing site) were synthesized *in vitro* and purified. The edited adenosine (*pA) at the 5' terminus of the 3'-half RNA was phosphorylated with polynucleotide kinase and [γ - 32 P]ATP and ligated using DNA ligase as described¹⁷. After editing, RNAs were recovered and digested with nuclease P1 to produce 5' monophosphates. **b**, TLC analysis. The 5' monophosphates produced from GluR-B RNA specifically labelled at the edited site (lanes 3, 4), a 5'-end-labelled 3'-half GluR-B RNA (lanes 5, 6) and a uniformly ([α - 32 P]ATP) labelled GluR-B RNA (lanes 7, 8), were analysed by one-dimensional TLC at 0 and 4 h. The novel nucleotide migrating closely with pI is labelled pI*. (It comigrated exactly with pI when adjusted for salt concentration.) **c**, Nucleotide pI*, produced from editing of the 3'-half GluR-B RNA labelled at the 5' adenosine (for 0, 2 and 6 h, as indicated), was analysed by two-dimensional TLC. **d**, Two-dimensional TLC analysis of the products of the editing reaction ('mix') are compared

(TLC) analysis revealed one faster-migrating nucleotide (pI*) which migrates most closely with inosine 5'-monophosphate (Fig. 2b, lanes 3 and 4; Fig. 2 legend). When the adenosine residues in GluR-B RNA were uniformly labelled, 0.5% (0.8% expected) of the adenosine produced by P1 digestion comigrated with pI*. To confirm the identity of the edited nucleotide, we examined the 5'-monophosphates from *in vitro* edited GluR-B RNA by two-dimensional TLC (Fig. 2c, d). Increasing amounts of the pI* product were seen with increasing times of incubation (Fig. 2c). The position of this product relative to 5'-monophosphate standards unambiguously identified pI* as inosine 5'-monophosphate (Fig. 2d).

We introduced mutations into the GluR-B RNA-editing substrate to determine whether the sequences required for GluR-B

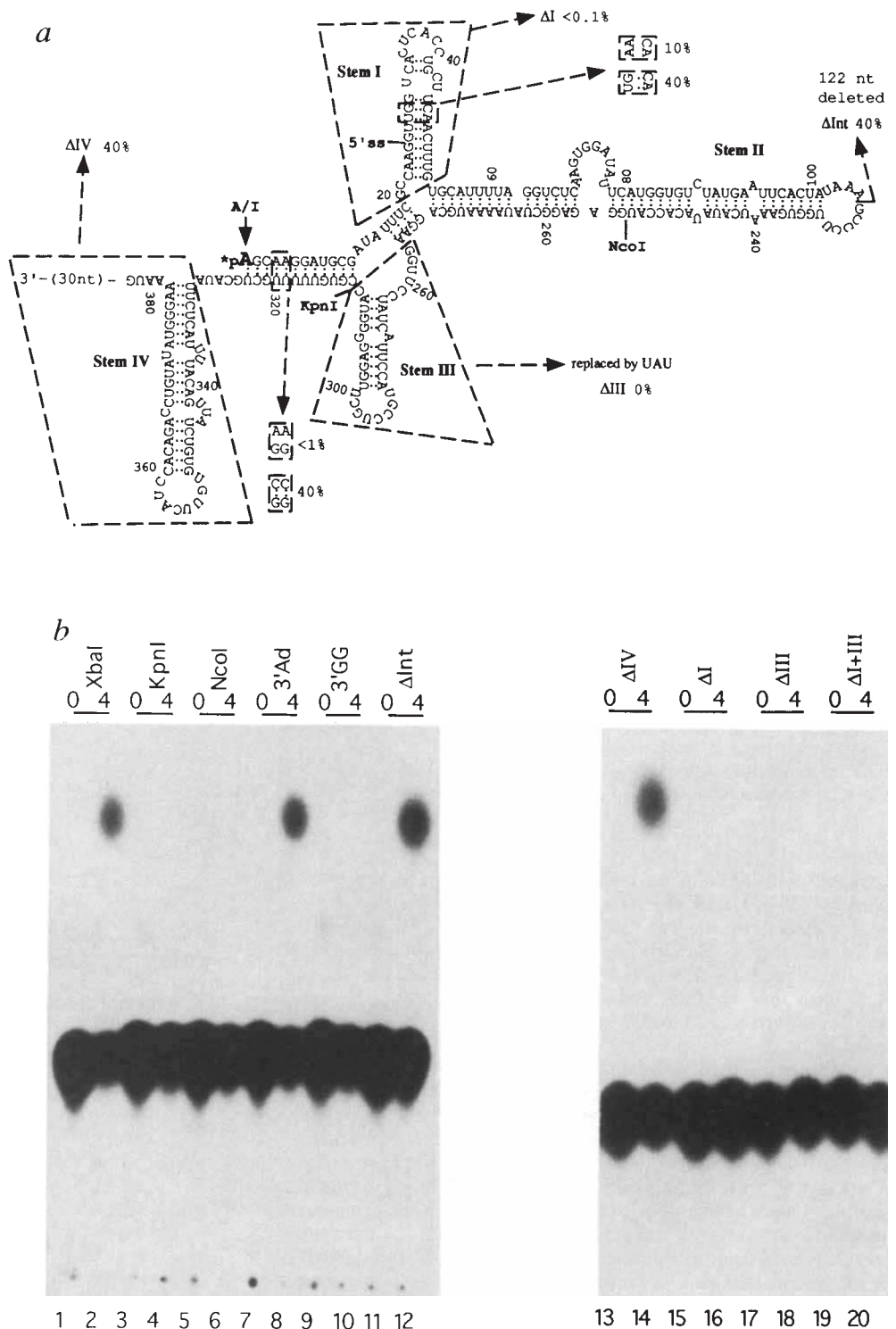
to standard 5' monophosphates. Left panel, all five standards; centre, three standards (pG, pC and pU) plus mix; right, two standards (pA and pI) plus mix.

METHODS. B169-208 is the bridging oligonucleotide used in the RNA ligation (GCGAAATATCGCATCCTTGCTGCATAAAGGCACCAAGGA). About 5 fmol ($1-5 \times 10^3$ c.p.m.) of labelled GluR-B RNA was incubated with HeLa cell nuclear extract. RNA was recovered by proteinase K treatment, phenol extraction and ethanol precipitation, and resuspended in 10 μ l of 5 mM Tris-HCl (pH 7.4), 1 mM EDTA and 0.5 U nuclease P1 (Sigma). After incubation at 50 °C for 3 h, digestion products were spotted onto a cellulose-PEI chromatography plate (J. T. Baker) and developed with butyric acid in 0.5 M NH_4OH (100:60 v/v) in the first dimension and saturated $(\text{NH}_4)_2\text{SO}_4$ in 0.1 M sodium acetate (pH 6.0) and isopropanol (79:19:2 v/v) in the second dimension. For one-dimensional TLC, only the second-dimension solvent was used. The nucleotides were visualized by autoradiography.

editing *in vitro* are the same as those required for editing *in vivo*⁸. The RNA sequence immediately upstream from the editing site has the potential for intrastrand base pairing with a complementary sequence in the intron⁸. Surprisingly, GluR-B RNA, which lacks this sequence, was edited as efficiently as full-length RNA (Fig. 2b, lanes 5 and 6). Although mutations that disrupt the base pairing immediately 5' to the editing site decrease the efficiency of editing *in vivo*⁸, complete deletion of this sequence had no effect in our *in vitro* system. We therefore used GluR-B RNA with the edited nucleotide at its 5' terminus (3' half GluR-B RNA) to assay the effects of other mutations on GluR-B RNA editing.

Several potential stem-loop structures surrounding the editing site are predicted by computational folding (Fig. 3a). The exon

FIG. 3 Sequence requirements for *in vitro* editing. **a**, Predicted secondary structure of the 3'-half GluR-B RNA. A/I indicates the edited nucleotide. Predicted RNA stem loops I to IV and restriction sites used to linearize corresponding DNA templates are indicated. The sequences surrounded by dashed-line boxes indicate deletions or substitution mutations. The editing efficiency (percentage adenosine converted to inosine) is indicated for each mutation. **b**, *In vitro* editing of mutants in the 3'-half GluR-B RNAs analysed by TLC. As shown, GluR-B RNA truncated upstream of the ECS (KpnI, NcoI, lanes 3–6) was not edited *in vitro*. But removal of stem loop IV (Δ IV, lanes 13–14) or addition of 100 nt at the 3' end of GluR-B RNA (3' Ad, lanes 7–8) had no effect on *in vitro* editing. Deletion of a predicted loop of 122 nucleotides between the two inverted repeats (Δ Int, lanes 11–12) had no effect on editing, as shown previously⁵. The same results were obtained for mutations in full-length GluR-B pre-mRNA (data not shown). Truncation mutants are indicated by the restriction endonucleases used to generate the DNA templates. Deletions of stem loops I, III and I+III together are indicated as Δ I, Δ III and Δ I+III, respectively. Notice that deletion of stem III or both stem I and III, with the addition of the three residues AUA, results in extension of the RNA duplex essential for editing by 7 or 16 base pairs, respectively. Simply extending this duplex did not improve GluR-B RNA editing. The 3' Ad template is the same as the XbaI template with an extra 32 nucleotides of vector sequence at its 3' end (from XbaI site to SP6 of pcDNAI/amp). The 3'GG template has ³¹⁹U³²⁰ replaced with ³¹⁹G³²⁰G. The Δ Int template has a deletion of 122 nucleotides from the intron sequence (106 to 227). **METHODS.** 3'-half GluR-B RNA was edited and analysed as for Fig. 2. All point mutations and deletions were made by PCR-mediated mutagenesis. RNA structure was predicted by MFOLD supplied in the GCG program package (Genetics Computer Group, Wisconsin).



complementary sequence (ECS) necessary for GluR-B editing *in vivo*⁸ is also required for editing in our extracts (Fig. 3b, lanes 1–6). Deletion of stem loop IV and a loop between the internal inverted repeats (Δ Int) had no effect on editing, but removal of stem loop I or III, or both, led to a complete loss of editing activity (Fig. 3b, lanes 7–20). Base substitutions at positions 28–29 within the consensus sequence of 5' splice site (from ²⁸U²⁹G to ²⁸A²⁹U), which partially disrupts the potential base pairing in stem II, reduced the editing efficiency from 41 to 10%, as assayed *in vitro* and *in vivo*. Conversion of the ³¹⁹U³²⁰U sequence to ³¹⁹G³²⁰G within the ECS region, which disrupts the putative RNA duplex spanning the editing site, abolishes *in vitro* editing

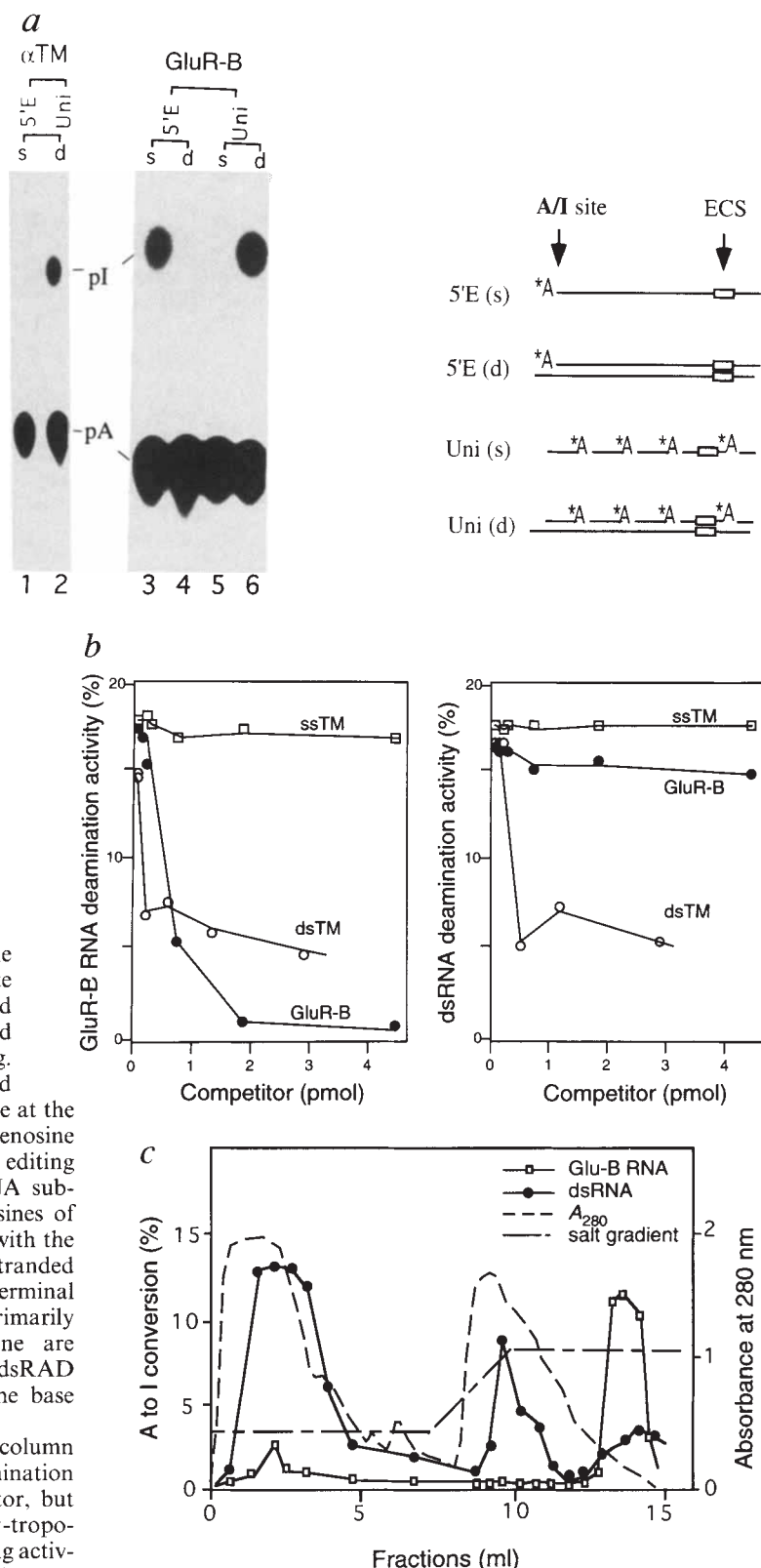
(3'GG), but a compensatory mutation with ⁴C⁵C downstream of the editing site, which restores the base pairing with the ECS, restores the editing activity to the level of wild-type RNA (data not shown). These results confirm the importance of the base-paired sequences within and downstream of the editing site, for site-specific GluR-B pre-mRNA editing *in vitro*.

We found that this *in vitro* editing activity does not require ATP or Mg²⁺; it is abolished by proteinase K and is unaffected by micrococcal nuclease. We conclude that GluR-B editing is accomplished by a protein without an essential RNA component. These properties are indistinguishable from those of the double-stranded RNA-dependent adenosine deaminase

FIG. 4 Differences between GluR-B editing and dsRNA-deamination activities. *a*, Comparison of the deamination activity of 5'-end-labelled (5'E) and uniformly 32 P-ATP labelled (Uni) 3'-half GluR-B RNA (GluR-B). RNA substrates used for the editing assay are shown on the right. Each substrate was examined as single-stranded RNA (s), or as a duplex formed between the labelled RNA and the corresponding unlabelled complementary RNA (d). As a control, the α -tropomyosin exon-2 RNA (α TM)²⁰ was tested for deamination of 5' terminal and internal adenosine residues and is referred to as dsRNA-deamination activity^{9,10,21}. *b*, RNA competition assay. Standard conditions for GluR-B RNA editing (left) and nonspecific dsRNA deamination (right, α TM dsRNA) were used for the competition assay in the presence of 0.02, 0.05, 0.2, 0.8, 2.0, 4.5 picomol unlabelled single-stranded α TM RNA (ssTM), double-stranded α TM RNA (dsTM), or single-stranded GluR-B RNA (GluR-B). The molar ratio between the labelled RNA and cold competitor RNA is 2, 5, 20, 80, 200 and 450, respectively. The relative activities are represented by percentage of A-to-I conversion in 4 h. *c*, HeLa nuclear extracts (4 ml) were loaded onto a poly(I)·poly(C)-agarose column (5 ml) and washed with buffer A (7.5 ml of 20 mM HEPES, pH 7.9, 5 mM EDTA, 50 mM KCl and 0.5 mM DTT). Proteins were eluted with a 4-ml gradient of KCl (50 mM KCl to 3 M KCl in buffer A) and eluted with 3 M KCl in buffer A (1.8 ml, indicated by dashed line). Fractions (0.5 ml) were collected, dialysed individually against buffer A containing 10% glycerol, and assayed for GluR-B editing activity with GluR-B RNA (GluR-B RNA; squares) and dsRNA-deamination activity with α TM dsRNA (dsRNA; filled circles). Absorbance at 280 nm is indicated by the dashed line; the salt gradient is shown as a broken line.

(dsRAD). To investigate this relationship between the editing activity and dsRAD, we tested the substrate requirements for the two activities. A double-stranded RNA substrate containing the editing site was produced by annealing 3' GluR-B RNA with its complement (Fig. 4a). Both this substrate and the normal single-stranded substrate were either labelled specifically at the adenosine at the editing site or were uniformly labelled. The terminal adenosine was efficiently edited in the single-stranded substrate, but editing was barely detectable in the fully double-stranded RNA substrate. By contrast, deamination of the internal adenosines of the uniformly labelled substrates was far more efficient with the double-stranded RNA substrate. Thus, the single-stranded GluR-B substrate is modified most efficiently at the terminal editing site, whereas the double-stranded RNA is primarily modified at internal adenosines. These results alone are insufficient to demonstrate that the GluR-B editing and dsRAD activities are distinct, because dsRAD does show some base selectivity on short dsRNA substrates¹⁸.

We therefore carried out substrate competition and column fractionation studies (Fig. 4b and c). The dsRNA-deamination activity was significantly inhibited by dsRNA competitor, but not by single-stranded GluR-B RNA or single-stranded α -tropomyosin exon-2 RNA (Fig. 4b). In contrast, GluR-B editing activity was inhibited by the specific single-stranded RNA or a nonspecific dsRNA. Thus, the dsRNA-deamination activity could not interact with GluR-B RNA specifically, although both activities appear to require the dsRNA-binding motif. In an attempt to separate the two activities we fractionated nuclear extracts on poly(I)·poly(C) agarose and monitored the GluR-B and dsRNA deaminase in each fraction. As shown in Fig. 4c, the two activity profiles are distinct. The dsRNA-deamination activity was detected mainly in fractions eluting at 50 mM KCl; a second peak was detected in the 50 mM to 3 M KCl eluate. A



relatively small amount of activity survived extensive washing with 3 M KCl. By contrast, GluR-B RNA editing activity was detected in a sharp peak that eluted only after washing with 3 M KCl. The two activities also fractionated as distinct peaks on Q-Sepharose and on GluR-B RNA immobilized on agarose (data not shown). Taken together, these observations indicate that GluR-B editing and dsRNA-deamination activities are distinct but that they may have dsRAD, or a similar deaminase, as a common component. For example, the GluR-B editing enzyme

may contain a sequence-recognition subunit that specifically targets a dsRNA-dependent adenosine deaminase to the secondary structure of GluR-B pre-mRNA. □

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A family of transcriptional adaptor proteins targeted by the E1A oncoprotein

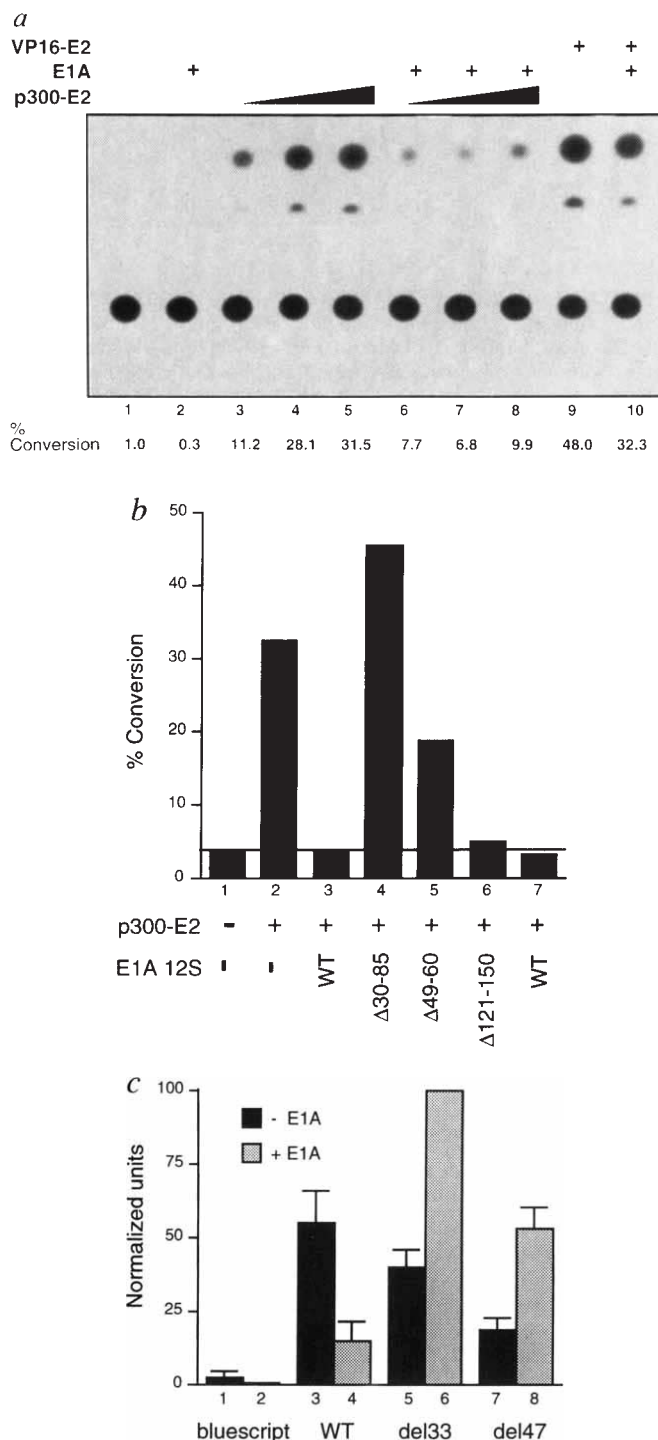
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THE cellular protein p300 is a target of the adenoviral E1A oncoprotein and is thought to participate in preventing the G0/G1 transition in the cell cycle, activating certain enhancers and stimulating differentiation pathways¹. CBP is a protein that is associated with and coactivates the transcription factor CREB, mediating the induction by cyclic AMP of certain responsive promoters^{2–4}. The sequences of p300 and CBP are highly related^{4,5}. We show here that p300, like CBP², can stimulate transcription. This activity is directly and specifically inhibited by E1A. We also find that CBP exists in a DNA-bound complex containing a member of the CREB family and that E1A and CBP interact with one another *in vivo*. In keeping with the idea that E1A functionally targets CBP, cAMP-dependent transcription is repressed by E1A. Thus, p300 and CBP define a family of transcriptional adaptor proteins that are specifically targeted by the E1A oncoprotein.

We have proposed that p300 is a coactivator which, when brought to DNA, can stimulate transcription⁶. To test this, p300, fused to the DNA-binding domain of the papillomaviral E2 protein, was cotransfected with an E2-responsive reporter plasmid

FIG. 1 p300–E2 stimulates transcription and is repressed by E1A. *a*, U-2 OS human osteosarcoma cells were cotransfected with 1 µg pCMV-lacZ; 3 µg tk6XE2-CAT¹⁸; 1, 2 or 6 µg pCMV-p300–E2 (lanes 3–5 and 6–8); and 2.5 µg pCMV-E1A12S, as indicated, and brought to 16 µg total with Bluescript DNA (Stratagene). *b*, U-2 OS cells were cotransfected with the reporter P2CAT (3 µg)¹⁹, with p300–E2 (6 µg), and, where indicated, with E1A plasmids bearing the indicated deletions (2.5 µg). They were assayed as in *a*. WT, wild type. E1A Δ30–85 is unable to interact efficiently with p300 (ref. 9); E1A Δ49–60 has greatly reduced affinity for p300 (ref. 8), E1A Δ121–150 interacts with p300 but not with any retinoblastoma-family protein²⁰. *c*, U-2 OS cells were transfected and assayed as in *a* and *b*. tk6XE2-CAT served as reporter, and pCMV-p300–E2 plasmids bearing the Δ33 (deleting amino acids 1,737–1,836), or Δ47 (1,676–1,750) deletions were included where indicated. METHODS. pCMV-p300–E2 was created by fusing the papillomavirus E2 DNA-binding domain¹⁹ to the C terminus of full-length, N-terminally haemagglutinin-tagged, human p300. Cells were transfected as described¹⁷, and chloramphenicol acetyltransferase (CAT) assays were performed 2 days later²¹ using extract quantities normalized to β-galactosidase activity²¹. Representative experiments are shown in *a* and *b*; *c* depicts the average of 3 experiments, with standard deviations indicated.



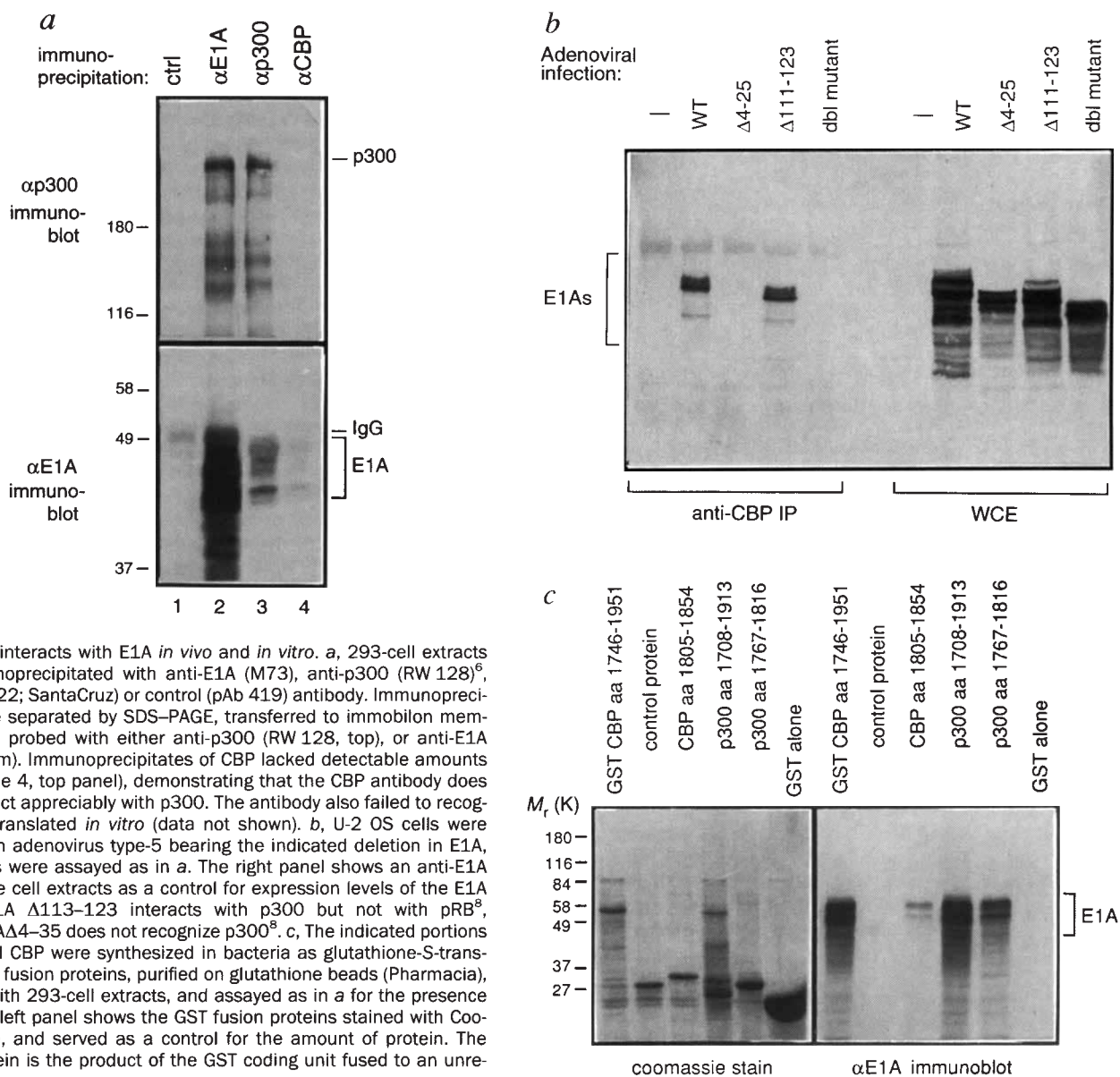


FIG. 2 CBP interacts with E1A *in vivo* and *in vitro*. **a**, 293-cell extracts were immunoprecipitated with anti-E1A (M73), anti-p300 (RW 128)⁸, anti-CBP (A-22; SantaCruz) or control (pAb 419) antibody. Immunoprecipitates were separated by SDS-PAGE, transferred to immobilon membranes, and probed with either anti-p300 (RW 128, top), or anti-E1A (M73, bottom). Immunoprecipitates of CBP lacked detectable amounts of p300 (lane 4, top panel), demonstrating that the CBP antibody does not crossreact appreciably with p300. The antibody also failed to recognize p300 translated *in vitro* (data not shown). **b**, U-2 OS cells were infected with adenovirus type-5 bearing the indicated deletion in E1A, and extracts were assayed as in **a**. The right panel shows an anti-E1A blot of whole cell extracts as a control for expression levels of the E1A proteins. E1A Δ 113-123 interacts with p300 but not with pRB⁸, whereas E1A Δ 4-35 does not recognize p300⁸. **c**, The indicated portions of p300 and CBP were synthesized in bacteria as glutathione-S-transferase (GST) fusion proteins, purified on glutathione beads (Pharmacia), incubated with 293-cell extracts, and assayed as in **a** for the presence of E1A. The left panel shows the GST fusion proteins stained with Coomassie blue, and served as a control for the amount of protein. The control protein is the product of the GST coding unit fused to an unrelated sequence.

METHODS. Plasmids encoding GST fusion proteins were created by amplification of the appropriate DNA fragments, which were then subcloned into pGEX2TK²². Transfections were done as in **a**. Infections were performed as described⁹ the day before cell lysis. Cell lysis, GST-

into U-2 OS human osteosarcoma cells. This gave a marked stimulation of reporter activity (Fig. 1a) which was reproducible on several promoters, including some in which the E2 sites were placed 3' to the reporter sequence (data not shown). Hence p300-E2 transactivates and does so with at least one hallmark enhancer property.

E1A 12S, when cotransfected in this assay, repressed p300-E2 activity (Fig. 1a). This repression is specific: inhibition by E1A mutants depended on their ability to interact efficiently with p300 (Fig. 1b). To confirm that this inhibition was a consequence of direct interaction with p300 (Fig. 1b). To confirm that this inhibition was a consequence of direct interaction with p300-E2, we created two deletions in p300-E2 which abolish E1A binding (ref. 6 and data not shown). E1A did not inhibit transactivation by either of these two proteins (Fig. 1c). Thus E1A specifically and directly inhibits p300-E2 activity.

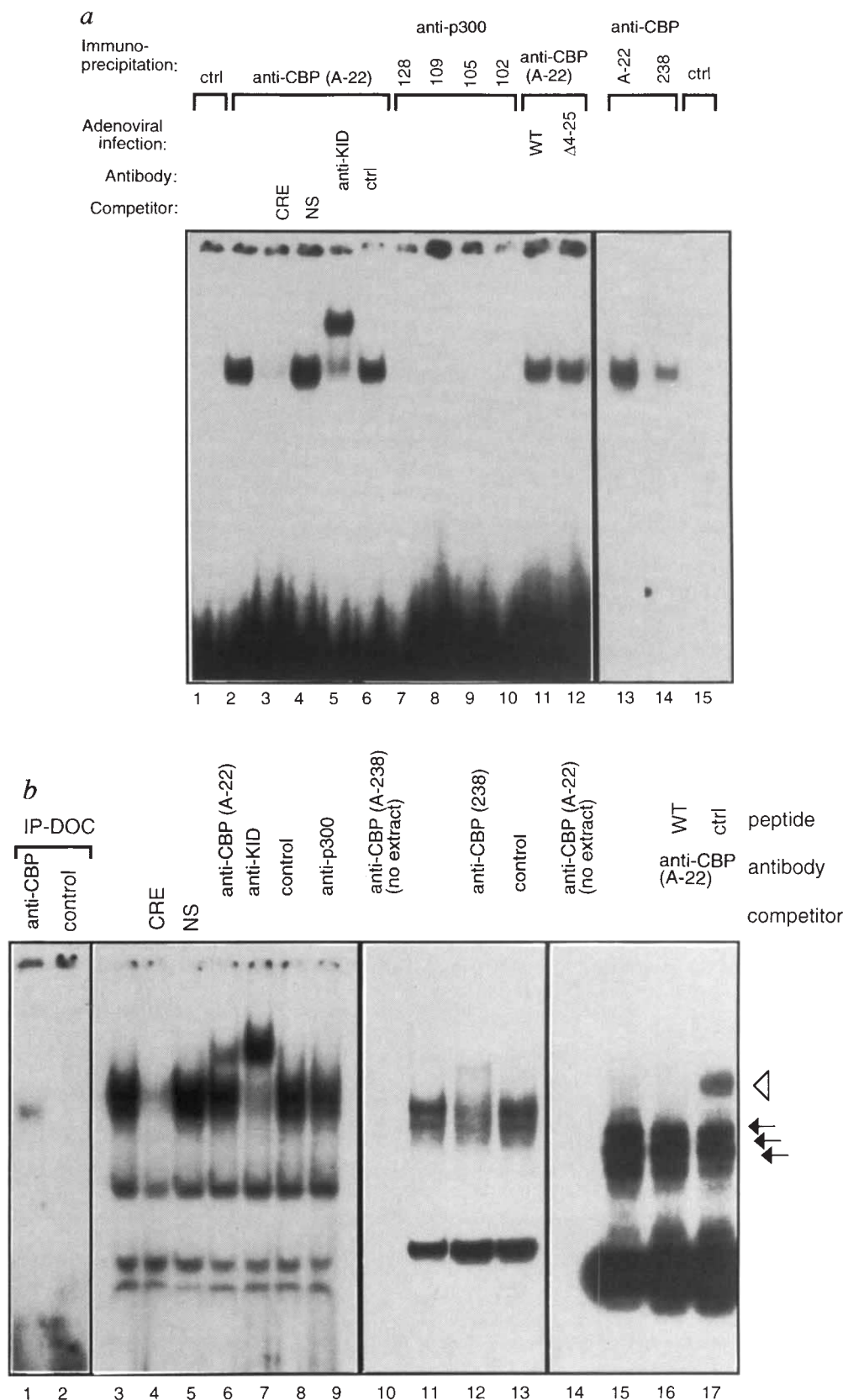
One deletion mutant, Δ 47, had reduced activity compared to wild-type p300-E2 (Fig. 1c). Interestingly, Δ 47 removes sequences homologous to the yeast ADA2 protein², a putative

fusion protein synthesis in *E. coli*, binding of GST proteins to glutathione beads, and incubation of loaded glutathione beads with cell extracts were done as described²². Immunoblots were developed using the alkaline phosphatase method (BioRad).

coactivator protein⁷. Therefore, this region may interact with important effectors of transactivation such as TFIIB³. In addition, E1A reproducibly stimulates the activity of the two mutants (Fig. 1c) without affecting protein levels (data not shown), suggesting that E1A also perturbs p300 function indirectly.

In view of the striking homology between p300 and CBP⁵, we tested whether E1A could bind CBP as well. We found that in cells expressing E1A constitutively, CBP and E1A did interact: anti-CBP immunoprecipitates contained proteins immunoreactive with E1A antibodies (Fig. 2a). To test the specificity of this interaction, we infected U-2 OS cells with adenoviruses bearing various deletions in E1A and assayed for E1A in anti-CBP immunoprecipitates. As shown in Fig. 2b, CBP interacts with the same E1A mutants as p300 (refs 8, 9), indicating that the same region of E1A recognizes both p300 and CBP. Conversely, E1A recognizes the same region in both p300 and CBP *in vitro*, namely the third cysteine/histidine-rich region (Fig. 2c). We conclude that CBP and E1A interact *in vivo* with the same specificity as do p300 and E1A.

FIG. 3 CBP interacts with a CREB family member *in vivo* and is found in a DNA-bound complex. **a**, U-2 OS cell extracts were immunoprecipitated with control (M73), anti-CBP (A-22, AC 238), or anti-p300 (RW 128, RW 109, RW 105, RW 102)⁶ antibodies, as indicated. Immunoprecipitates were eluted with deoxycholate (DOC), and eluates were assayed by electrophoresis mobility shift (EMSA) with a CRE-containing probe (Promega). 20-fold excess of cold, specific (lane 3) or nonspecific (NS: Myc E2F site, lane 4) probe was used as competitor. Anti-KID antibody (anti-ATF-1-125C10G; SantaCruz), which recognizes CREB family members, and control antibody (anti-mouse immunoglobulin, Cappel) were analysed as potential supershifting reagents in lanes 5 and 6, respectively. Extracts in lanes 11 and 12 were prepared from cells infected with adenovirus type-5 encoding wild-type E1A or E1A deleted of residues 4–35, respectively. Polyclonal antibody A-22 was raised against a peptide of CBP amino acids 2–22 (SantaCruz); monoclonal antibody AC 238 was raised against a GST fusion protein containing CBP amino acids 720–1,676 (R.E. and D.M.L., unpublished results). **b**, U-2 OS extracts were assayed directly by EMSA using the same probe as in **a**. Anti-KID, control, anti-CBP (A-22, AC 238) and anti-p300 (RW 128) antibodies were analysed as potential supershifting reagents, as indicated. As controls, anti-CBP antibodies were reacted with the probe alone in lanes 10 and 14. The anti-CBP (A-22) antibodies used in lanes 16 and 17 were pre-incubated with the immunogenic peptide (SantaCruz) or control peptide, respectively. The data presented in the left two panels are from a single gel, and the samples loaded in lanes 1 and 2 are the same samples as loaded in lanes 2 and 1 in **a**, respectively. **METHODS.** Extracts, immunoprecipitations, DOC elutions, and EMSA assays were performed as described²³, except for the gel conditions in the fourth panel of **b**, which were performed according to ref. 24. Infections were done as in Fig. 2. Preincubations of anti-CBP antibodies were carried out overnight, at 4 °C, with 10-fold excess by weight of peptide.



We next investigated, using electrophoresis mobility shift assays, whether CBP could be brought to certain cAMP-responsive promoters by interacting directly with one or more CREB-family members^{2,3}. As shown in Fig. 3a, deoxycholate eluates of anti-CBP immunoprecipitates contain a specific cAMP response element (CRE)-binding activity (lanes 2–4, 14) generated by a complex containing a CREB-family member (lane 5). Consistent

with the idea that E1A inhibits CBP transactivation but not CREB binding, infection of the cells with adenovirus before lysis had no effect on the observed complex (Fig. 3a, lanes 11 and 12). Whole-cell extracts yielded at least three specific complexes (Fig. 3b, lanes 3–5, 11, 15), the fastest of which comigrated with the complex present in the anti-CBP immunoprecipitation eluates (lanes 1 and 3). All three complexes were supershifted

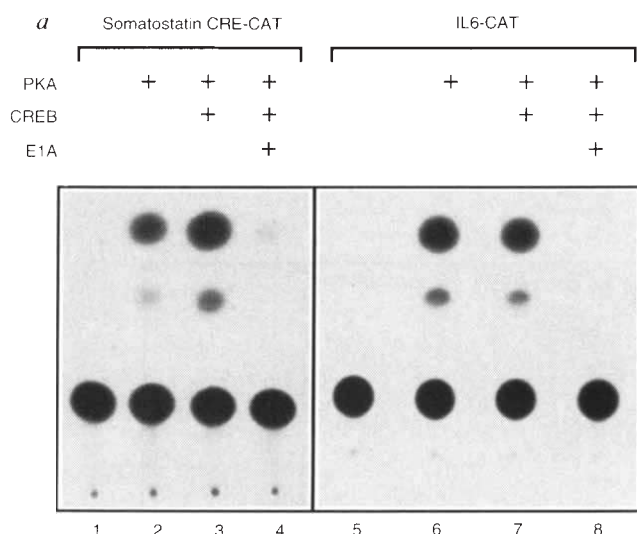
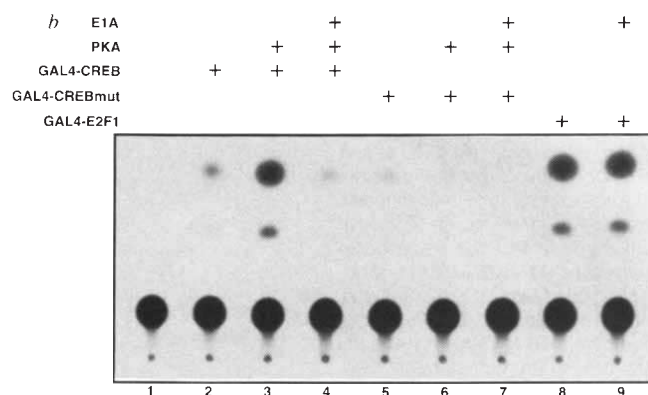


FIG. 4 E1A inhibits CRE-mediated and CREB-mediated transactivation. **a**, U-2 OS cells were transfected with 1 μ g pCMV-lacZ, 3 μ g somatostatin CRE-CAT²⁵, 3 μ g IL6-CAT²⁶, 5 μ g pCEV_{neo}²⁶, 3 μ g RSV- Δ CREB, and/or 2.5 μ g pCMV-12S E1A, as indicated. Where necessary, Bluescript DNA (Stratagene) was added to each transfection mixture to bring the DNA input to 16 μ g. **b**, U-2 OS cells were transfected with 1 μ g pCMV-lacZ, 5 μ g GAL4-CAT²⁷, 3 μ g pCMV-GAL4 CREB, 3 μ g pCMV-GAL4 CREBmut, 5 μ g pCEV_{neo}²⁶, 1 μ g GAL4 E2F-lac368–437 Δ 412–416 (as control)²⁷, and/or 2.5 μ g pCMV-12S E1A, as indicated.

by an antibody recognizing CREB family members (lane 7), and one or more of them contained CBP, because addition of anti-CBP antibodies generated a slower-migrating complex (Fig. 3b, lanes 6, 12, and 16, 17). By contrast, all p300 antibodies tested failed to bind these complexes (Fig. 3a, lanes 7–10; Fig. 3b, lane 9, and data not shown). Taken together, our results show that CBP interacts *in vivo* with at least one CREB family member as a specific DNA-bound complex. Interestingly, these complexes are present even in the absence of active transcription (Fig. 4a, lanes 1 and 5).

What is the consequence of the interaction of E1A with CBP? Figure 4a shows that in U-2 OS cells E1A inhibits cAMP-dependent activation of the minimal somatostatin promoter and of the intact interleukin-6 promoter; this latter observation, made in other cells, has already been reported¹⁰. Figure 4b shows that E1A inhibits the cAMP-dependent activity of the CREB transcription factor itself: a GAL4-CREB fusion protein, brought to a promoter by a GAL4 site, is repressed by E1A. We conclude that E1A can downregulate transcriptional activation mediated by CREB and, as others have suggested^{10,11}, at least some CREs. On the basis of our results, we suggest that this



Bluescript DNA was added as in **a**.

METHODS. Transfections and CAT assays were performed as in Fig. 1. All cells were incubated in the presence of 80 μ M ZnCl₂ to induce, where appropriate, synthesis of protein kinase A (PKA), which is driven by the metallothionein promoter. pCMV-GAL4 CREB^{mut} bears a mutation in serine 119 which abolishes cAMP-dependent transactivation²⁸. pCMV-GAL4 CREB and pCMV-GAL4 CREBmut were created by subcloning the inserts from plasmids pZ1 and pZ1A^{mut28}, respectively, into pcDNA3 (Invitrogen).

inhibition is a consequence, at least in part, of direct interaction with and inhibition of CBP.

We have shown that p300, like CBP², has an intrinsic transcription activation function. Furthermore, this activity is downregulated directly and specifically by E1A. E1A can target CBP *in vivo*, and CBP in turn associates with one or more CREB-family members as part of a stable, DNA-bound complex. Hence, a CBP/E1A interaction provides at least one mechanistic explanation for the observation that E1A inhibits the activity of various CREs (Fig. 4a and refs 10, 11) and of CREB itself (Fig. 4b). The ability of E1A to interfere directly with an integral part of the cAMP pathway could be important in light of the well documented growth-inhibitory and differentiation-inducing effects of cAMP^{12–15}. Although the effects on cell growth are in part independent of transcription^{16,17}, promotion of cell differentiation is regulated by transcription factors responsive to cAMP^{14,15}. E1A, by targeting CBP, might inhibit these same factors and induce the opposite phenotype. Taken together, our results argue that E1A may deregulate growth and differentiation partly by inhibiting a family of transcriptional coactivators. □

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Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP

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THE 265K nuclear protein CBP was initially identified as a co-activator for the protein kinase A (PKA)-phosphorylated form of the transcription factor CREB¹. The domains in CBP that are involved in CREB binding and transcriptional activation are highly related to the adenoviral E1A-associated cellular protein p300 (refs 2, 3), and to two hypothetical proteins from *Caenorhabditis elegans*, R10E11.1 and K03H1.10 (refs 4 and 5, respectively), whose functions are unknown. Here, we show that CBP and p300 have similar binding affinity for the PKA-phosphorylated form of CREB, and that p300 can substitute for CBP in potentiating

CREB-activated gene expression. We find that E1A binds to CBP through a domain conserved with p300 and represses the CREB-dependent co-activator functions of both CBP and p300. Our results indicate that the gene repression and cell immortalization functions associated with E1A involve the inactivation of a family of related proteins that normally participate in second-messenger-regulated gene expression.

The high degree of similarity (Fig. 1a) between p300, R10E11.1 and K03H1.10 suggests that they may have related functions. To test whether p300 and R10E11.1, like CBP, could bind to PKA-phosphorylated CREB, amino-terminal fragments of each protein were expressed in bacteria and assayed for binding by far-western analysis. All three proteins bound to the phosphorylated CREB probe (Fig. 1b), suggesting that all have the potential to participate in the regulation of CREB-modulated genes through an evolutionarily conserved domain. Interruption of the conserved, highly charged carboxy-terminal portion of the CREB-binding domain of CBP greatly reduces phospho-CREB binding (Fig. 1b; CBP(451–661)).

The phospho-CREB interaction with CBP and p300 was analysed quantitatively using a fluorescence-polarization binding assay^{6,7} to determine whether the two putative co-activators have different binding properties. The homologous CBP and p300 domains interact with phospho-CREB with identical affinity, and show the previously reported^{1,6} specificity for the PKA-phosphorylated form of CREB (Fig. 1c).

To test directly whether p300 can participate in cyclic AMP-regulated gene expression, we used a functional assay devised

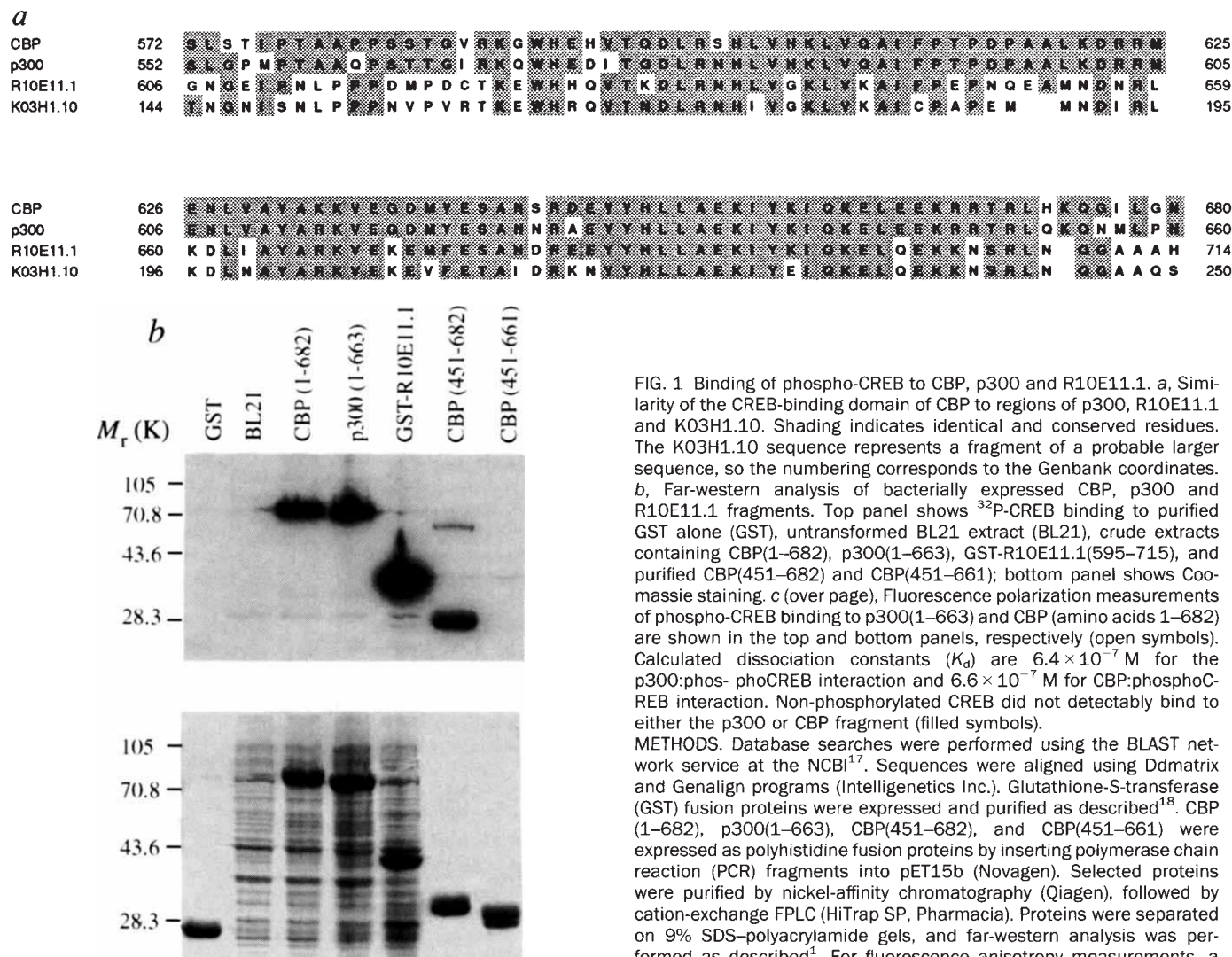


FIG. 1 Binding of phospho-CREB to CBP, p300 and R10E11.1. **a**, Similarity of the CREB-binding domain of CBP to regions of p300, R10E11.1 and K03H1.10. Shading indicates identical and conserved residues. The K03H1.10 sequence represents a fragment of a probable larger sequence, so the numbering corresponds to the Genbank coordinates. **b**, Far-western analysis of bacterially expressed CBP, p300 and R10E11.1 fragments. Top panel shows ³²P-CREB binding to purified GST alone (GST), untransformed BL21 extract (BL21), crude extracts containing CBP(1–682), p300(1–663), GST-R10E11.1(595–715), and purified CBP(451–682) and CBP(451–661); bottom panel shows Coomassie staining. **c** (over page), Fluorescence polarization measurements of phospho-CREB binding to p300(1–663) and CBP (amino acids 1–682) are shown in the top and bottom panels, respectively (open symbols). Calculated dissociation constants (K_d) are 6.4×10^{-7} M for the p300:phospho-CREB interaction and 6.6×10^{-7} M for CBP:phospho-CREB interaction. Non-phosphorylated CREB did not detectably bind to either the p300 or CBP fragment (filled symbols). **METHODS.** Database searches were performed using the BLAST network service at the NCBI¹⁷. Sequences were aligned using Ddmatrix and Genalign programs (Intelligenetics Inc.). Glutathione-S-transferase (GST) fusion proteins were expressed and purified as described¹⁸. CBP (1–682), p300(1–663), CBP(451–682), and CBP(451–661) were expressed as polyhistidine fusion proteins by inserting polymerase chain reaction (PCR) fragments into pET15b (Novagen). Selected proteins were purified by nickel-affinity chromatography (Qiagen), followed by cation-exchange FPLC (HiTrap SP, Pharmacia). Proteins were separated on 9% SDS-polyacrylamide gels, and far-western analysis was performed as described¹. For fluorescence anisotropy measurements, a

(FIG. 1 continued) fluorescein-labelled CRE (5 nM) was incubated with saturating amounts of CREB or phospho-CREB, then incubated with increasing concentrations of purified N-terminal fragments of CBP or p300⁶. Anisotropy values for each titration point were determined using a BEACON Fluorescence Polarization system (Pan Vera Corp.). Binding isotherms were fitted to a simple binding model for the interaction of CBP or p300 with phospho-CREB, including a linear nonspecific binding component, by nonlinear regression. Protein concentrations were confirmed by amino-acid analysis.

previously to assess the activity of CBP⁶. CBP has been shown to augment the transcriptional activity of CREB in F9 teratocarcinoma cells in a way that depends on the presence of both CREB and PKA⁶. Here we tested whether p300 could substitute for CBP in this assay. As shown in Fig. 2, p300 also augments the transcriptional activity of CREB and PKA in a dose-dependent manner. This function of p300 requires the presence of PKA-phosphorylated CREB, as a mutant CREB protein (M1) lacking the PKA phosphorylation site does not allow p300 to activate expression of the reporter.

p300 was initially identified through its ability to co-immunoprecipitate with E1A^{8,9}, and the sequences in p300 responsible for this interaction have been localized to the C-terminal zinc-finger motif². This region is highly conserved in CBP (where it allows interaction with the basal transcription factor TFIIB⁶), suggesting that CBP may also associate with E1A. Because of the extensive homology between CBP and p300, it has not been possible to distinguish the two proteins in E1A-associated protein complexes. We found that an antipeptide antibody directed against the N-terminal 22 amino acids of CBP¹ is highly specific (Fig. 3a). Immunoblot analysis of E1A-associated proteins immunoprecipitated from adenovirus-transformed 293 cells demonstrates that both CBP and p300 associate with E1A *in vivo* (Fig. 3b). The interaction with CBP does not require post-translational modifications of E1A, as shown by the ability of an E1A monoclonal antibody to immunoprecipitate complexes of purified recombinant 12S E1A and epitope-tagged CBP

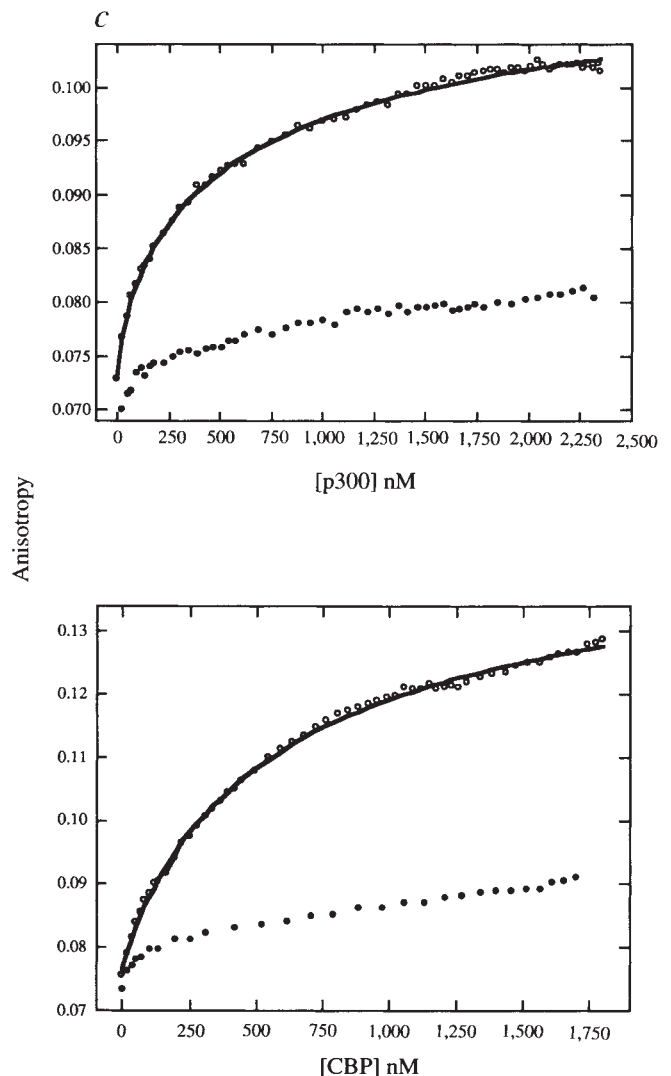


FIG. 2 p300 potentiates CREB-activated gene expression. F9 teratocarcinoma cells were transiently transfected with p(-71)SRIF-CAT¹⁹, pRSV-luciferase, pRc/RSV-PKA, pRc/RSV-CREB341, and pRc/RSV-p300. In some experiments, a form of CREB containing a serine-to-alanine mutation at position 133 (pRc/RSV-CREBM1) was used⁶. The results are expressed as relative chloramphenicol acetyltransferase (CAT) activity (mean $\pm$ s.e., $N=4$) compared to the CAT activity in the absence of CREB. CAT activity values are normalized for luciferase activity. METHODS. F9 transfection has been described⁶. Each transfection experiment contains 2 μ g pRSV-luciferase, 5 μ g p(-71)SRIF-CAT, 4 μ g pRc/RSV-PKA, 4 μ g pRc/RSV-CREB341 or 4 μ g pRc/RSV-CREBM1, and various amounts of p300. pRc/RSV-p300 was constructed from the plasmid pCMV/ β -p300 (ref. 3).

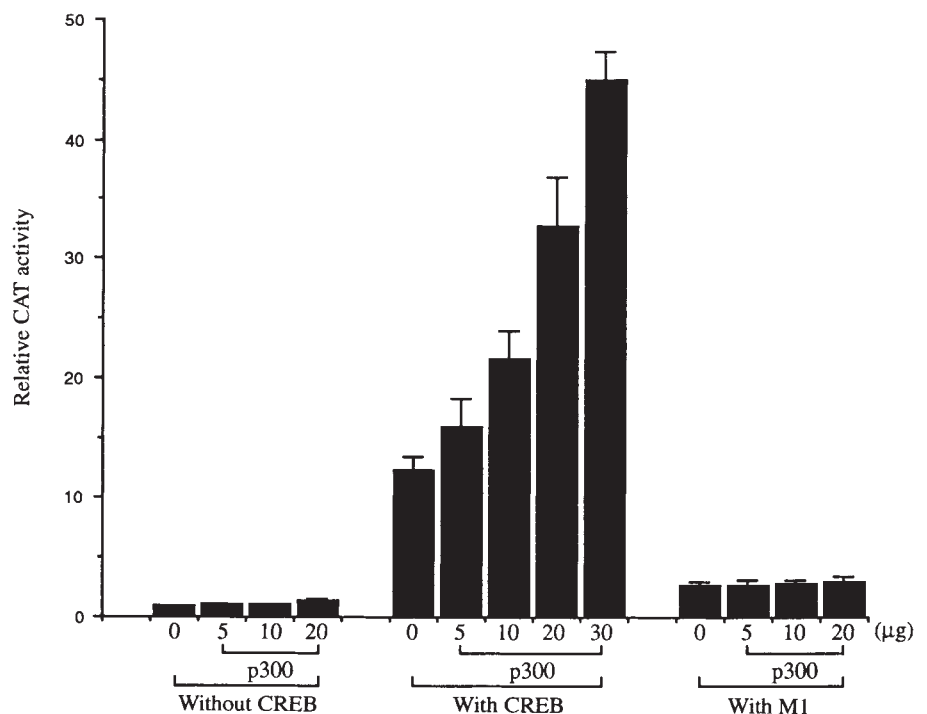
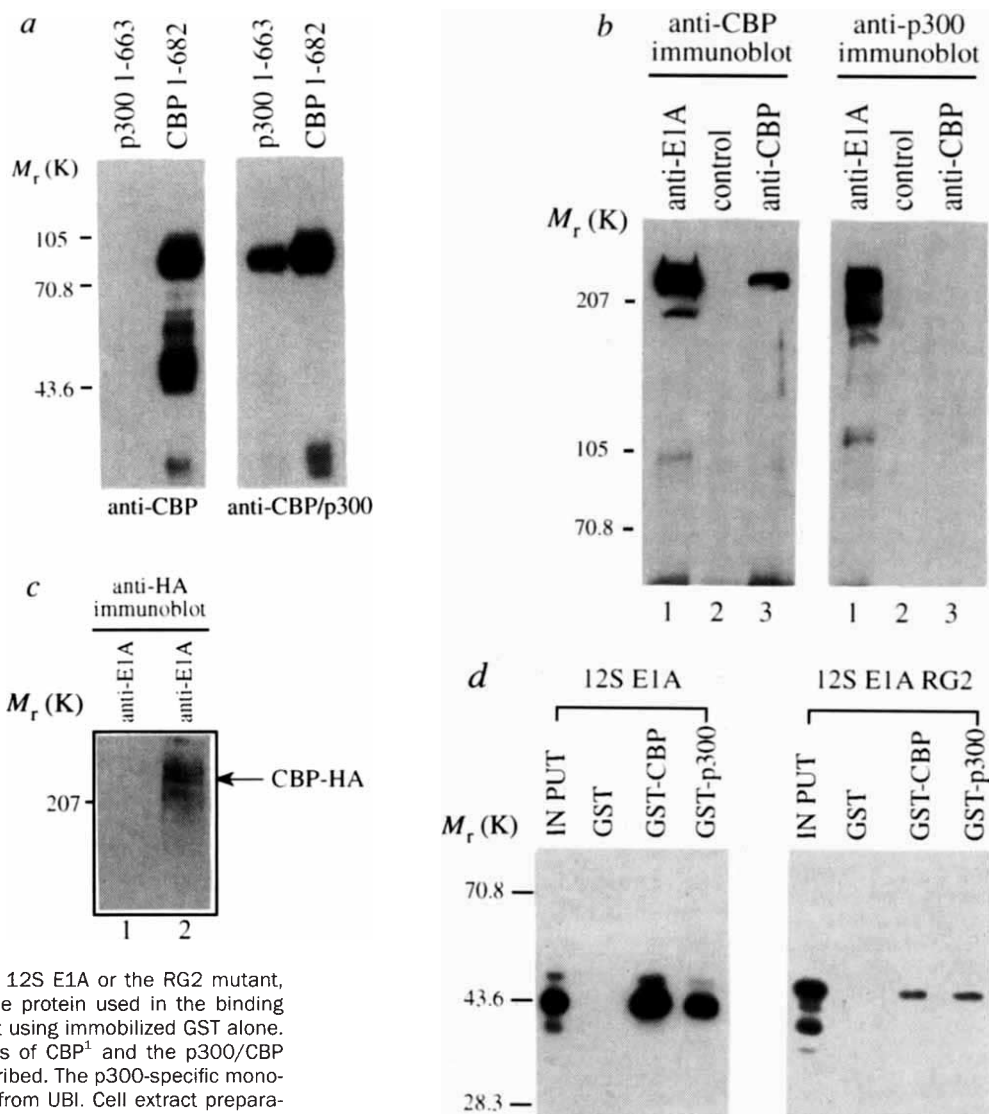


FIG. 3 CBP association with E1A. *a*, Immunoblot analysis of bacterially expressed N-terminal fragments of CBP and p300. CBP(1–682) and p300(1–663) were electrophoretically separated, transferred to PVDF membrane, and detected with either a specific N-terminal CBP antipeptide antibody¹ (anti-CBP; left) or an antipeptide antibody directed against the conserved CREB-binding domains of CBP and p300¹³ (anti-CBP/p300; right). *b*, Association of CBP with E1A *in vivo*. Whole-cell lysates from 293 cells were immunoprecipitated with the E1A-specific monoclonal antibody M73 (ref. 8; see lane 1), control antibody (FLAG M1; lane 2) or the CBP-specific antibody (lane 3). Immune complexes were then analysed by western blotting with the specific CBP N-terminal antibody (left) or a p300-specific antibody (UBI, 05-257; right). *c*, Binding of E1A to epitope-tagged CBP in cellular extracts. Lane 1 contains extract from F9 cells transfected with control expression vector; lane 2 contains extract from cells transfected with haemagglutinin (HA)-tagged-CBP expression vector. Extracts were mixed with purified recombinant E1A and immunoprecipitated with antibody M73. Positions of CBP-HA and the 207K size marker are indicated. *d*, Binding of 12S E1A to GST fusion proteins. GST fusion proteins encoding CBP(1,680–1,891) or p300 (1,642–1,856) were immobilized on glutathione-agarose

beads and incubated with recombinant 12S E1A or the RG2 mutant, as indicated. Input refers to 20% of the protein used in the binding assays. GST refers to control experiment using immobilized GST alone. METHODS. Antibodies to the N-terminus of CBP¹ and the p300/CBP CREB-binding domain¹³ have been described. The p300-specific monoclonal antibody (05-257) was obtained from UBI. Cell extract preparation and conditions for immunoprecipitations and immunoblot analysis have been described^{9,20}. To detect association of recombinant E1A with CBP-HA, purified E1A (200 ng) was added to lysates (750 µg) from F9 cells transfected with control expression vector pcDNA3 (lane 1) or pCMV-CBP-HA (lane 2). M73 immune complexes were analysed using anti-HA monoclonal antibody (12CA5; Boehringer Mannheim) and visualized by ECL (Amersham). pCMV-CBP-HA was constructed by inserting an oligonucleotide encoding the HA epitope at the C terminus of the CBP coding region and ligating the fusion cDNA into pcDNA3 (Invitrogen).



Expression and purification of 12S E1A protein is described elsewhere²¹. For the GST binding assay, equal amounts of GST, GST-p300 and GST-CBP were coupled to glutathione-agarose beads in 10 mM HEPES, pH 7.4, 200 mM NaCl, 0.5 mM EDTA, 0.5 mM DTT, 0.1% NP-40, with 5 mg ml⁻¹ BSA. 12S E1A and RG2 proteins were incubated with the immobilized GST fusion proteins for 1 h at room temperature, washed, and the bound proteins were eluted with SDS-PAGE loading buffer. E1A was detected using M73 antibody and ECL.

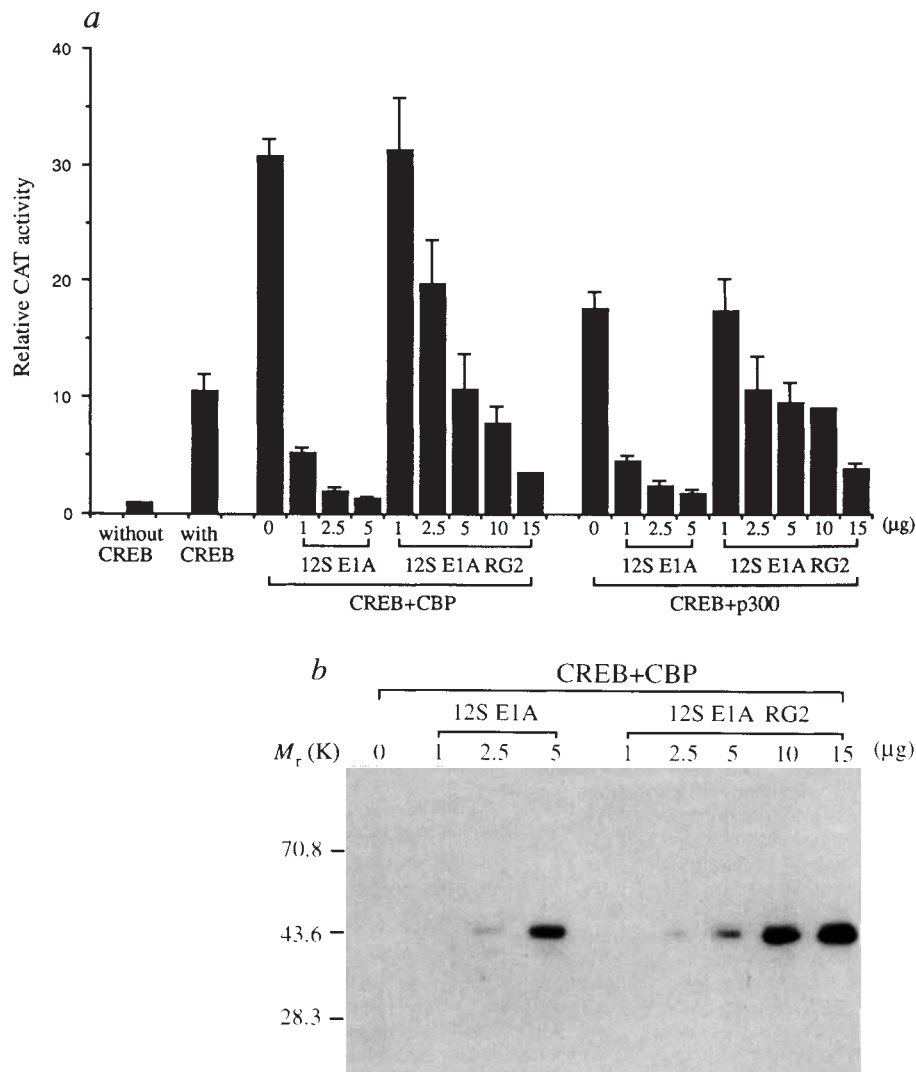
expressed in F9 cells (Fig. 3c). Finally, a direct interaction between E1A and the third zinc-finger motif of CBP was determined using glutathione-S-transferase (GST) fusion proteins (Fig. 3d). The RG2 mutant E1A protein, which contains an arginine-to-glycine substitution in the second position and interacts poorly with p300 (ref. 10), also binds less efficiently to CBP and p300 fusion proteins (Fig. 3d).

The induction of genes by cAMP and phorbol esters is blocked by 12S E1A^{11,12}. To test whether this effect could be mediated through CBP and/or p300, we used the functional assay already described. F9 teratocarcinoma cells were transfected with CREB, PKA, and an amount of CBP or p300 vector sufficient to provide half-maximal expression of a CRE reporter⁶. These experiments indicated that 12S E1A inhibits CBP and p300 function in a dose-dependent manner (Fig. 4a). The E1A mutant RG2 inhibits CBP and p300 function less efficiently, even when corrected for the amount of E1A protein produced

in these transfection experiments (Fig. 4b).

CBP and p300 thus appear to have very similar functional properties. The observation that p300 can activate cAMP-induced gene expression does not preclude its participation in other pathways, however. For example, overexpression of p300 can overcome E1A-repression of the SV40 enhancer². Also, antibody blocking experiments suggest that CBP may co-activate phosphorylated c-Jun¹³. The repressive transcriptional effects of 12S E1A are therefore probably due to interference at the co-activator level. Although generally a repressor of gene transcription, 12S E1A also activates transcription of a subset of genes in a TATA-specific manner¹⁴. This transactivation likewise seems to require the N-terminal p300/CBP binding region of E1A¹⁵. But it is not known whether CBP/p300 participates in transcriptional activation through 13S E1A, because this pathway appears to require conserved region 3, which is absent in the 12S form¹⁶. Nonetheless, our results indicate that E1A-

FIG. 4 Inhibition of CBP and p300 activities by 12S E1A. *a*, F9 cells were transiently transfected with p(-71)SRIF-CAT, pRSV-luciferase, pRc/RSV-PKA and mixtures of pRc/RSV-CREB341, pRc/RSV-CBP, pRc/RSV-p300, pRc/RSV-E1A 12S and pRc/RSV-E1A 12S RG2 as indicated. The results are expressed as the relative CAT activity (mean $\pm$ s.e., $N=4$) compared to the CAT activity in the absence of CREB (without CREB column). CAT activity values are normalized for luciferase activity. *b*, Western blot analysis of the relative levels of 12S E1A and 12S E1A RG2 proteins expressed in the transfection experiments in *a*. METHODS. 4 μ g of pRc/RSV-CREB341, 15 μ g pRc/RSV-CBP, 20 μ g pRc/RSV-p300 and various amounts of E1A expression vectors were used in each transfection. The PCR products used to construct pRc/RSV-12S E1A and Rc/RSV-12S E1A RG2 were confirmed by sequencing. Equal amount of proteins from each sample of the transfection experiments were separated on 10% SDS-polyacrylamide gels, transferred onto nitrocellulose, and blocked with 5% non-fat dry milk in TBS buffer with 0.1% Tween-20. Proteins were detected using M73 antibody and ECL.



induced cell immortalization may depend, in part, on interactions between the viral-transforming protein and a family of cellular transcription co-activators. □

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A highly conserved ATPase protein as a mediator between acidic activation domains and the TATA-binding protein

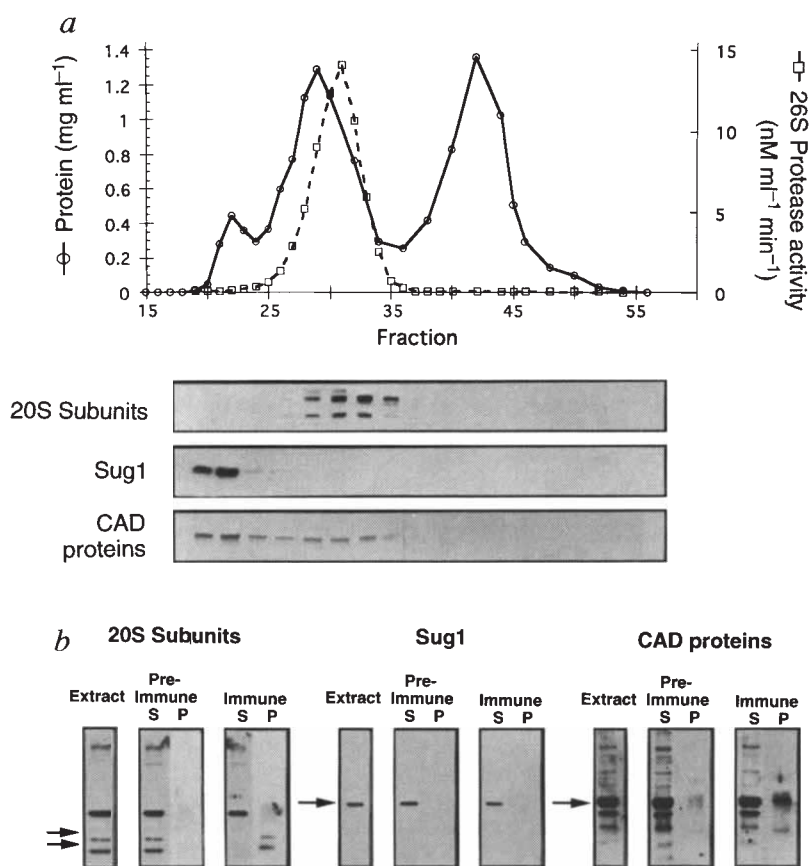
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BIOCHEMICAL and genetic studies suggest the existence of mediators that work between the activation domains (ADs) of regulatory proteins and the basic transcriptional machinery. We have previously shown genetically that Sug1 interacts with the AD of the yeast activator Gal4'. Here we provide evidence that the Sug1 protein of yeast binds directly to the ADs of Gal4 and the viral activator, VP16. Sug1 protein is associated with the TATA-binding protein *in vivo* and binds to it *in vitro*, consistent with a mediator function. We also demonstrate that Sug1 is not a compo-

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FIG. 1 Sug1 is not present in the 26S proteasome. **a**, Sug1 physically separates from the 26S proteasome upon gel filtration. A yeast whole cell extract was passed down a gel filtration column. Fractions were collected and assayed for total protein, 26S proteasomal activity, Sug1, 20S proteasomal subunits and Sug1-related CAD proteins. **b**, Sug1 does not coimmunoprecipitate with the 26S proteasome. The 26S proteasome was immunoprecipitated from a yeast WCE. Supernatants (S) and pellets (P) were then assayed for the presence of Sug1, 20S proteasomal subunits, and Sug1-related CAD proteins. We judge the procedure to precipitate complete 26S proteasome particles based on the ability of the proteasomal activity of the precipitate to be stimulated by ATP to the same extent as the WCE (data not shown). Only intact 26S proteasomes are capable of stimulation by ATP^{11,28,29}. **METHODS.** *Saccharomyces cerevisiae* strain W303 in which the only copy of *SUG1* had been tagged at the N terminus with the T7 coat protein S10 epitope (NOV-AGEN) was grown in YEP galactose. Cells were collected, and a WCE made by lysing protoplasts in an equal volume of column buffer (20 mM TRIS pH 7.6, 20 mM K-acetate, 1 mM EDTA, 1 mM dithiothreitol (DTT), 20% v/v glycerol, 170 $\mu\text{g ml}^{-1}$ PMSF, 0.7 $\mu\text{g ml}^{-1}$ pepstatin A, 0.5 $\mu\text{g ml}^{-1}$ leupeptin) minus the glycerol. The now viscous solution was sonicated for 5–10 s on ice and spun at 35,000g for 45 min at 4 °C. The clear supernatant was removed, avoiding the upper lipid layer, and glycerol added to 20% v/v. Extracts typically contained 25–35 mg ml^{-1} protein. The solution was diluted to 20 mg ml^{-1} and 8 ml loaded onto a 2.5 $\times$ 100 cm Sephacryl S-400 (Pharmacia) column (bed volume ~450 ml). Fractions (8 ml) were collected at a flow rate of 24 ml h^{-1} and analysed. Protein was determined by the Bradford assay (Biorad). 26S proteasomal activity was assayed against the synthetic peptide Suc-Leu-Leu-Val-Tyr-AMC (7-amido-4-methylcoumarin) (Bachem) as previously described³⁰. Fractions were probed by western blot for 20S subunits (polyclonal rabbit antibody supplied by K. Tanaka), Sug1-like CAD proteins (rabbit polyclonal antibody raised to *E. coli* produced Sug1), S10-Sug1 (anti-T7(S10) monoclonal antibody obtained from Novagen). For immunoprecipitations,



ment of the 26S proteasome, contrary to previous reports^{2–5}. Sug1 is a member of a large, highly conserved family of ATPases, implying a role for ATP hydrolysis in the activation of transcription.

SUG1 was identified genetically by a mutation, *sug1-1*, that rescued defects in the *Gal4 AD*¹. On the basis of genetic studies we proposed that Sug1 was a candidate for a transcriptional mediator and that, unlike *Ada2*⁶ and *Gal11*^{7,8} (two other mediator candidates), the role of Sug1 was essential because a deletion was lethal. Recently, however, several other highly related Sug1-like proteins (CAD proteins, for conserved ATPase-domain containing) have been identified as components of the 26S proteasome^{2–5,9,10}. This has led to the suggestion that Sug1 is not a transcriptional factor, as we originally hypothesized, but a protein that affects the turnover of transcriptional factors².

We directly determined whether Sug1 is a proteasomal component by isolating the 26S proteasome and assaying specifically for Sug1 in the complex. There has been no reported fractionation of the 26S form of the proteasome from yeast so we adapted a protocol used to isolate the mammalian complex¹¹. All of the proteasome activity eluted from a size-fractionation gradient in a peak centred around $\sim 2 \times 10^6 \text{K}$, the expected size of the intact 26S complex. This identification was confirmed by probing each fraction in western blots with antibodies (K. Tanaka) raised against a subcomplex of the 26S proteasome, the 20S subunit. As expected, a peak of 20S proteins coincided with the 26S activity peak. Under these extraction conditions the 26S form of the yeast proteasome was quite stable and there was no evidence of dissociation to release free 20S subunits (no free 20S

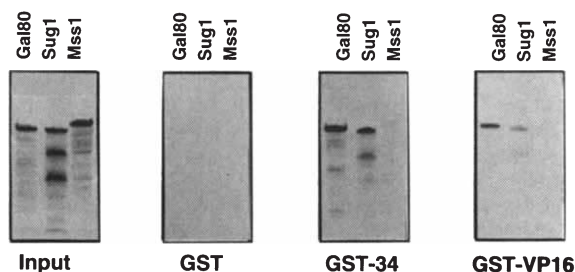
subunits (M_r 700K) or SDS stimutable proteasome activity in fractions 35–40)¹². Each fraction was then probed for the presence of proteins recognized by an anti-Sug1 polyclonal antibody that crossreacts with related CAD proteins. Two peaks were present. One corresponded to the 26S peak and the other to a larger peak centred around greater than $3 \times 10^6 \text{K}$. To determine which of these peaks contained Sug1 we introduced an epitope tag (bacteriophage T7-S10) onto the amino terminus of Sug1. This tagged protein fully substitutes for the essential function of Sug1 when introduced into a strain deleted for the endogenous *SUG1* gene (data not shown). When the same fractions were probed for the S10 epitope, Sug1 is only detectable in the larger fraction and is not present in the 26S peak.

This conclusion was confirmed by an independent assay. Conditions were established under which the 20S antibody immunoprecipitated all of the 26S protease activity from the crude extract, with no dissociation of the complex (as revealed by the ATP stimulation of the precipitated complex). Under these conditions all of the 20S proteins are precipitated but no Sug1 is coprecipitated. However, CAD proteins were coprecipitated with the 20S proteins (Fig. 1b). These two sets of experiments indicate that Sug1 is not a component of the proteasome but that other CAD proteins are.

Having demonstrated that Sug1 is not proteasomal we investigated its presumed role in transcription. We first addressed whether Sug1 protein physically interacts with the AD of Gal4 and the other prototypic acidic AD, that of the mammalian virus, VP16. Glutathione-S-transferase (GST) fusions were made to the ADs of Gal4¹³ (GST-34) and VP16¹⁴ (GST-VP16).

FIG. 2 Sug1 binds directly to the activation domains of Gal4 and VP16. *In vitro* translated and labelled Gal80, Sug1 and Mss1 were incubated with GST, GST-34 (Gal4 amino acids 841–875) and GST-VP16 (the full bi-partite VP16 AD fused to GST). Input lanes represent 20% of total labelled protein.

METHODS. GST fusion proteins were produced in *E. coli* strain BL21 (DE3) (pLysS) from the pGEX vector and bound to glutathione-agarose beads (Pharmacia). The protein loading was estimated by BCA protein assay (Pierce) after boiling the agarose beads in 1% SDS for 5 min. 35 S-methionine-labelled Sug1, Mss1 and Gal80 were produced by *in vitro* translation (Promega rabbit reticulocyte lysate) using standard protocols with the addition of 5 mM caffeine. Labelled proteins were incubated with agarose beads loaded with 5 μ g GST or GST-fusion proteins, in bead buffer (50 mM K-phosphate pH 6.0, 150 mM KCl, 10 mM MgCl₂, 10% Glycerol, 1% Tween-20) containing 10 mg ml⁻¹ soluble *E. coli* protein. For the binding assay, *E. coli* extract in bead buffer was centrifuged at 70,000 r.p.m. for 30 min in a Beckman TL-100. The supernatant was removed and 400- μ l aliquots placed in screw-capped Eppendorf tubes. Glutathione beads and labelled protein were added and the tubes



nutated gently at 4 °C for 1 h. The beads were pelleted at 13,000 r.p.m. for 1 min in a microfuge and washed 3 times with 1 ml bead buffer. The beads were finally spun down, all the supernatant removed, 20 μ l SDS loading buffer added and the samples boiled for 5 min. The labelled proteins bound to the beads were analysed by 10% SDS-tricine PAGE. Input lanes represent 20% of total input.

Both ADs specifically bind *in vitro* translated Sug1 protein (Fig. 2). Mss1 protein is not retained and serves as a negative control for specificity. Mss1 is a highly related member of the CAD protein family, having 62% amino-acid identity to Sug1 in the ~220 amino-acid sequence defining this family. Mss1 is a component of the human 26S proteasome³ and is functionally interchangeable with its yeast proteasomal homologue⁵. Gal80 (a negative regulatory protein known to bind directly the Gal4 AD)^{13,15} is the positive control. That Gal80 binds the VP16 AD may reflect structural similarities between these activation domains. The Sug1-AD interaction can be reproduced with purified, rather than *in vitro* translated, Sug1 protein (S. Russell and S.A.J., unpublished results), obviating the possibility that the binding relied on factors in the translation mixture.

Having established a physical *in vitro* interaction between Sug1 and ADs, we wished to know if Sug1 associates with other transcription factors *in vivo*. To address this question, gel filtration of a yeast lysate was done under the same conditions described for Fig. 1a, and the fractions probed in western blots for various transcriptional and non-transcriptional proteins. All proteins tested which are directly involved in transcription (TFIIB, RNA polymerase II, Srb4, Ada2 and Gal4) eluted with a profile similar to that of Sug1, whereas other proteins did not (Gal6, Cit1). TATA-binding protein (TBP) also eluted with the same profile as Sug1 (Fig. 3a), therefore we focused on their relationship. Sug1 was immunoprecipitated from the peak fractions from the gel filtration column and the immunoprecipitate probed by western blot for TBP. A substantial proportion, but

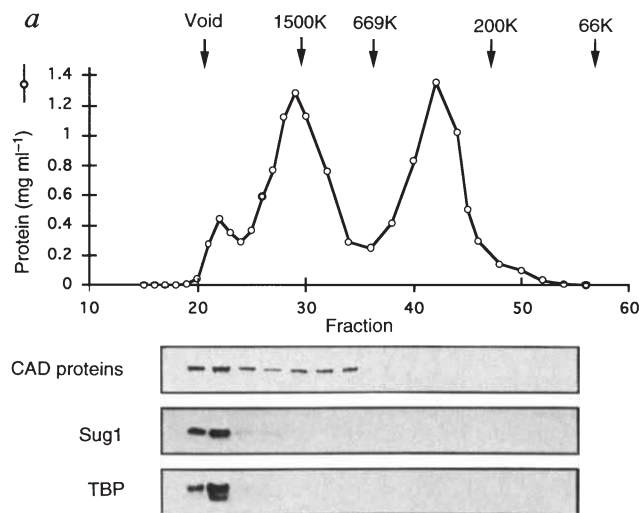


FIG. 3 Sug1 interacts directly with TBP. a, TBP elutes from a gel filtration column with the same profile as Sug1. Repeated passage down the sizing column of the Sug1-TBP containing fractions results in the same elution profile suggesting that this complex does not represent non-specific aggregation. Sug1 and TBP coimmunoprecipitate from a S400 gel filtration column fraction. b, Sug1 and TBP coimmunoprecipitate from an S400 gel filtration column fraction. c, Sug1 binds directly to TBP *in vitro*. *In vitro* translated Sug1, Gal80 and Mss1 were incubated with GST, GST-34 and GST-TBP (full-length yeast TBP fused to GST). Input lanes represent 20% of total input.

METHODS. Fractions were obtained and probed by western blot as described in Fig. 1. Of the fraction containing S10-Sug1 obtained from the S400 gel filtration column detailed in Fig. 1, 400 μ l was pre-cleared by adding 50 μ l unbound *S. aureus* cells and nutating for 2 h at 4 °C. The *S. aureus* cells were spun down and the supernatant used for

the immunoprecipitations. *S. aureus* cells (10 μ l), loaded with rabbit polyclonal anti-T7 (S10) antibodies (from J. Keene), were added to 400 μ l precleared fraction, nutated 1 h at 4 °C, washed 3 times in 1 ml column buffer, and the pellet taken up in either 1 $\times$ SDS loading buffer, or in NET for storage. Immunoprecipitated protein was visualized by 10% SDS-tricine PAGE followed by western blotting. Extract lanes represent 66% of the total input to the reaction. *In vitro* binding assays were as described in the legend to Fig. 2.

not all, of the TBP coprecipitates with Sug1 (Fig. 3b). The fact that only a portion of the TBP is precipitated may reflect an intrinsically weak association between the two proteins, that some TBP is engaged with RNA polymerase I and RNA polymerase III or that only a subset of Sug1 is associated with TBP at any time.

The coimmunoprecipitation of Sug1 and TBP indicates that Sug1 and some portion of TBP are components of the same complex *in vivo*. To test for a direct interaction between these two proteins we used the *in vitro* binding assay described above. Purified GST-TBP and GST-34 fusion proteins, as well as GST itself, were incubated with *in vitro* translated and labelled Gal80, Mss1 and Sug1 proteins. Figure 3c shows the results of these experiments and indicates that Sug1 can indeed bind directly to TBP. On a molar basis, GST-TBP retained ~25% of the amount of labelled Sug1 retained by GST-34.

This work establishes two points. First, it extends the confirmed roles of CAD proteins in basic cellular functions to transcription. CAD proteins have been found in archaeobacteria¹⁶, prokaryotes¹⁷ and eukaryotes and have been shown to be essential components of the cellular machinery for secretion¹⁸, peroxisomal¹⁹ and mitochondrial^{20,21} function and the proteasome^{2-5,9,10}. The CAD region is highly conserved (>50% identity in eukaryotes) in all these proteins, with the highest conservation between the proteasomal and transcriptional CADs. Yet the proteasomal Mss1 will not functionally substitute for the transcriptional Sug1 even when overexpressed (unpublished data). Though they are not functionally interchangeable, transcriptional and proteasomal CAD proteins can both arise in the same genetic selection⁵, possibly because protein levels can be altered either by modifying the rate of degradation (proteasome) or production (transcription). We have noted in comparing the CAD proteins that it may be possible to distinguish proteasomal from transcriptional proteins by their estimated isoelectric points (4.5–5.6 versus 8–9.5, respectively).

Second, we provide one of the few examples combining genetic and physical evidence for a mediator function. Recently two 'holo-complexes' containing RNA polymerase II have been isolated^{22,23}. These holo-complexes contain some of the same transcription factors as those that fractionate with Sug1 and one has been shown to include Sug1²². Both of these holo-complexes are smaller (1.2×10^6 K) than our Sug1-containing fraction. This may be due to the association of DNA with our complex or because there are more transcription factors associated with it. Note that the Sug1-containing complex of ref. 22 did not contain TBP, suggesting that our complex may contain more components.

Given that Sug1 physically interacts with both Gal4 AD and TBP, the simplest interpretation is that Sug1 is a 'linker' between enhancer-binding proteins and the basal complex. However, the fact that Sug1 is a predicted ATPase suggests that its role may be more than architectural, and that by analogy to the function of the Sec18/NSF CAD protein, may even involve the disassembly¹⁸ or rearrangement²⁴ of transcriptional complexes.

Mediators may be specific for certain genes or function universally. The latter type should be essential and highly conserved. Sug1 is essential in yeast and recently its human homologue, Tripl, was isolated²⁵. Tripl interacts with the AD of thyroid receptor in a ligand-dependent fashion. It functionally substitutes for Sug1 in yeast. It shares 86% amino-acid identity over the CAD region and 76% overall identity, making it the most highly conserved transcription factor isolated to date. In comparison, human and yeast TBP share only 65% overall identity and 81% identity in the conserved C-terminal domain. Moreover, human and yeast TBP are not functionally interchangeable *in vivo*^{26,27}. Tripl interacts with the acidic ADs of Gal4 and VP16 and the non-acidic AD of thyroid receptor, implying that Sug1-like mediators interact with diverse activators and that they play a role in the functions of most if not all RNA polymerase II promoters. □

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Interaction of thyroid-hormone receptor with a conserved transcriptional mediator

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THE thyroid-hormone receptors are hormone-dependent transcription factors that control expression of many target genes^{1,2}. This regulation is presumably a consequence of hormone-dependent contacts between the receptors and the basal transcription machinery³. We used the yeast two-hybrid system^{4,5} to identify a candidate human transcriptional mediator that interacts with both the thyroid-hormone receptor and the retinoid-X receptor in a ligand-dependent fashion. This protein, Tripl (for thyroid-hormone-receptor interacting protein), shares striking sequence conservation with the yeast transcriptional mediator Sug1 (refs 6, 7). Here we show that Tripl can functionally substitute for Sug1 in yeast, and that both proteins interact *in vitro* with the thyroid-hormone receptor, and with the transcriptional activation domains of yeast GAL4 and of herpes virus VP16.

We have identified several human proteins that interact with the thyroid-hormone receptor TR-β1 (ref. 8). In the yeast two-

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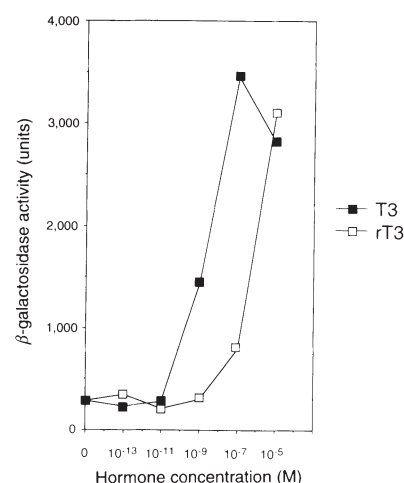
FIG. 1 Ligand-dependent interaction of Trip1 and TR in yeast. A derivative of EGY48 in which β -galactosidase expression is dependent on the presence of a transcriptional activator with a LexA DNA-binding domain⁵ was transformed with plasmids expressing LexA-TR and B42-Trip1 chimaeras, grown overnight in liquid medium containing the indicated concentrations of either T_3 (3,3',5'-triiodothyronine) or rT_3 (3,3',5'-triiodothyronine), and β -galactosidase activity was measured. In the presence of LexA-TR alone, saturating doses of T_3 induce a level of β -galactosidase expression less than 5% of that observed in the presence of B42-Trip1 (ref. 10). Expression of the B42 chimaeras is under the control of the GAL1 promoter⁵, and increased β -galactosidase expression dependent on B42-Trip1 is also dependent on the presence of galactose, as expected.

METHODS. Hybrid proteins were expressed from vectors that include the full-length LexA and B42 sequences, as described⁵. LexA-TR contains rat TR- $\beta 1$ (ref. 9) sequences from amino acids 169–461 (ref. 10). The Trip1 chimaera, one of the initially isolated cDNA clones, includes amino acids 49–406. Similar results were obtained with all of the other B42-Trip1 chimaeras, although some, including the full-length protein, showed low but significant β -galactosidase expression in the absence of hormone.

hybrid system, the thyroid hormone T_3 strongly stimulates the interaction of the most frequent isolate, Trip1, with the thyroid-hormone receptor (TR). When an appropriate yeast strain coexpressing LexA-TR and B42-Trip1 hybrids was grown in the presence of increasing doses of T_3 , half-maximal induction of expression of the β -galactosidase reporter gene was observed at a concentration consistent with the affinity of TR- $\beta 1$ for the hormone⁹ (Fig. 1). As expected, about 100-fold more reverse T_3 (rT_3) was required for half-maximal expression, and there was no response to glucocorticoids. T_3 elicited only a small increase in β -galactosidase expression in the absence of expression of the B42-Trip1 chimaera¹⁰.

B42-Trip1 also interacts with a LexA-retinoid-X-receptor fusion (LexA-RXR) in a way that depends on the amount of 9-*cis*-retinoic acid, but does not interact with an analogous glucocorticoid-receptor (GR) chimaera in the presence or absence of glucocorticoids (results not shown). Neither does B42-Trip1 interact with LexA alone, nor with several other LexA fusions to transcription factors or other proteins.

Trip1 is encoded by ~1,400-nucleotide messenger RNA which is expressed in all the human and other mammalian tissues and cell lines we examined (results not shown). Its amino-acid



sequence (Fig. 2a) shows that it is a member of an expanding superfamily of proteins that contains a domain of ~200 amino acids associated with ATPase activity. Among these CAD (for conserved ATPase domain) proteins, which are involved in diverse cellular functions, Trip1 shows particularly striking similarity to the essential yeast factor, Sug1 (ref. 6), with 86% amino-acid identity in the CAD region and 76% over the full length of both proteins (Fig. 2b). The less conserved Trip1 sequences N-terminal to the CAD region are absent in several of the B42-Trip1 chimaeras initially isolated (Fig. 2a) and are also dispensable for the direct *in vitro* interaction of Trip1 with Tr described later (not shown).

Sug1 is a transcriptional mediator, as shown by the ability of Sug1 mutations to suppress the effects of mutations in the activation domain of GAL4 (ref. 6) and the identification of Sug1 as a component of an RNA polymerase II holoenzyme complex, termed the mediator, which confers responsiveness to the effects of upstream activators *in vitro*¹¹. As described in the accompanying letter⁷, Sug1 interacts specifically *in vitro* with both transcriptional activation domains and the TATA-box-binding protein TBP.

Trip1 is less closely related to the human MSS1 protein¹² and other CAD protein components of a large protease complex¹³. In disagreement with previous suggestions^{4,15}, Sug1 is not a component of this complex⁷. Trip1 and Sug1 do not seem to be related to a protein of relative molecular mass 160K that binds the oestrogen receptor in a ligand-dependent manner¹⁶.

Based on their remarkable sequence similarity, we considered that Trip1 might functionally substitute for Sug1 in yeast. As shown in Table 1, Trip1 behaves like the wild-type *SUG1* allele in partially suppressing the transcriptional effects of a mutant *sug1-1* allele, whereas MSS1 has no effect. To determine whether Trip1 could also complement the lethal effect of loss of Sug1, a Trip1 expression plasmid was introduced into a yeast strain in which *SUG1* expression is controlled by the glucose-repressible *GAL* promoter. As shown in Fig. 2c, cells expressing Trip1, but not those transformed with vector alone, can grow on glucose-containing media. MSS1 cannot compensate for the loss of *SUG1* expression, although it can complement a mutation in another yeast CAD gene¹⁵. MSS1 also fails to interact with LexA-TR in the two-hybrid system (not shown). Thus, Trip1 and Sug1 are functionally equivalent and distinct from MSS1.

As shown in Fig. 3a, full-length TR can bind specifically to a Trip1-glutathione-S-transferase (GST) fusion protein and to a GST-Sug1 fusion. RXR is also retained by both Trip1 and Sug1, but GR, luciferase and other control proteins are not (not shown). Trip1 and Sug1 also bind to the activation domains of both GAL4 (GST-34) and VP16, but the proteasomal MSS1

TABLE 1 Sug1 and Trip1 partially suppress the rescue of *sug1-1* by *gal4D*

Genotype		β -Galactosidase (%)
<i>SUG1</i>	<i>GAL4</i>	100
<i>SUG1</i>	<i>gal4D</i>	3
<i>sug1-1</i>	<i>GAL4</i>	117
<i>sug1-1</i>	<i>gal4D</i>	55
<i>sug1-1</i>	<i>gal4D</i> pVT	50
<i>sug1-1</i>	<i>gal4D</i> pVT-SUG1	14
<i>sug1-1</i>	<i>gal4D</i> pVT-Trip1	27
<i>sug1-1</i>	<i>gal4D</i> pCHO-MSS1	50

Congenic yeast strains YJOZ (*SUG1*) and YJOZS (*sug1-1*) were transformed with either pSBGAL4 or pSBgal4D (centromeric yeast plasmids expressing GAL4p from its own promoter at physiological levels). Single colonies were then transformed with a series of expression plasmids as indicated. Double transformants were selected and β -galactosidase assays performed in minimal glycerol/lactic acid media as described⁶. β -Galactosidase levels in the *SUG1*, *GAL4* strain were set to 100% and correspond to 2280 Miller units (nmol o-nitrophenyl- β -D-galactopyranoside hydrolysed per min per mg soluble protein). The confidence limits of the assays are as follows: *SUG1 gal4D*, $n = 7$, s.d. = 0.4; *sug1-1 GAL4*, $n = 6$, s.d. = 14; *sug1-1 gal4D*, $n = 7$, s.d. = 9; *sug1-1 gal4D* pVT, $n = 4$, s.d. = 6.5; *sug1-1 gal4D* pVTSUG1, $n = 4$, s.d. = 2.5; *sug1-1 gal4D* pVTTrip1, $n = 10$, s.d. = 2.5; *sug1-1 gal4D* pCHO-MSS1, $n = 5$, s.d. = 6.3.

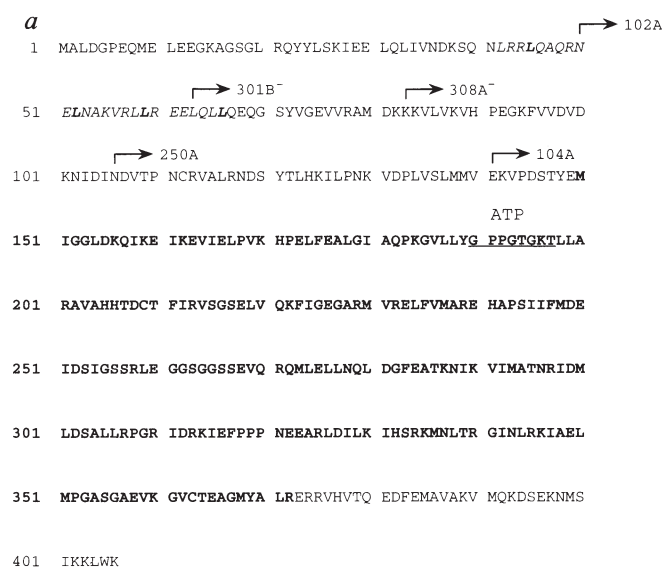
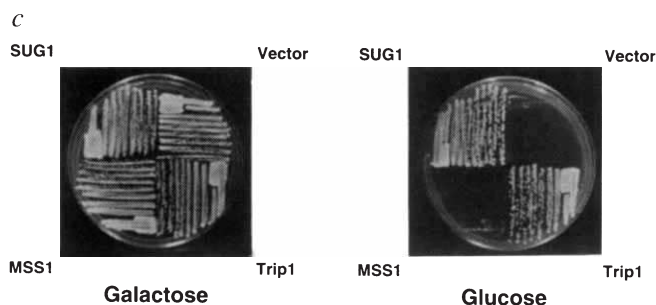
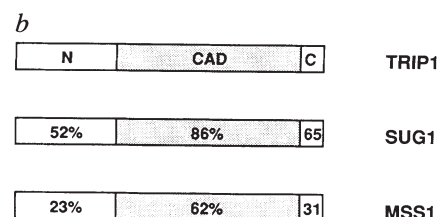


FIG. 2 **a**, Trip1 amino-acid sequence. The positions of the fusion junctions in 5 independent B42-Trip1 isolates are indicated by arrows, and a potential leucine zipper is indicated in italics. The conserved ATPase domain (CAD) is in bold type, and the potential ATP-binding site in this region is underlined. **b**, Comparison of Trip1 to two closely related CAD family proteins, Sug1 (ref. 6) and MSS1 (ref. 12). The CAD region is shaded, and the percentage of amino acids identical to those in Trip1 is shown for each protein. The alignment for the Trip1-Sug1 comparison did not include any gaps. Gaps were introduced into the Trip1-MSS1 comparison to maximize similarity. **c**, Trip1 can suppress the lethal deletion of *SUG1* in yeast. The yeast strain W303ASUG1::URA3 carrying a single copy of the *SUG1* gene under the control of the GAL1/10 promoter in the plasmid pMTL, was transformed with yeast plasmids



pYEP112, pYEP112 expressing the Trip1 gene under control of the yeast ADH1 promoter, pYEP112 expressing *SUG1* under control of the ADH promoter, or pRS314 expressing MSS1 under the control of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. Transformants were patched onto galactose and glucose plates. MSS1 expression in the *SUG1* mutant strain was confirmed by western blotting.

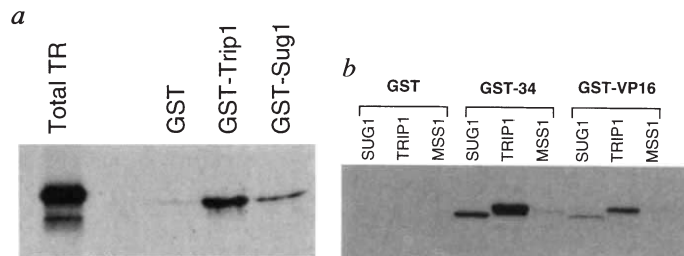
METHODS. Trip1 DNA sequence (Genbank accession number, L38810) was determined from 5 independently isolated B42 chimaeras. Fragments containing apparently full-length Trip1 N-terminal and 5' untranslated sequences were obtained by PCR from two separately generated *Hela* cDNA libraries, using primers from the plasmid vectors and from Trip1 sequences.

does not (Fig. 3b). Thus, both Trip1 and Sug1 directly and specifically interact with TR, GAL4 and VP16. In contrast to the results in yeast, however, *in vitro* interaction between Trip1 and the full-length TR protein is T_3 -independent. Preliminary results with fragments of TR indicate a ligand-independent interaction of Trip1 with N-terminal TR sequences not present in the LexA-TR fusion, and a stimulatory effect of ligand on the interaction of Trip1 with the TR ligand-binding domain. Further analysis will be required to define the nature of these interactions precisely.

Trip1 has several features expected for a mediator of TR-regulated transcription. It is a functional homologue of the yeast mediator Sug1, and interacts in a T_3 -dependent fashion with a region of TR containing the T_3 -dependent transcriptional activation domain. Like Sug1, Trip1 also interacts *in vitro* with the VP16 and GAL4 transcriptional activation domains. In contrast to the apparent lack of sequence similarity of these targets, Trip1 and Sug1 share even more sequence similarity than the highly conserved yeast and human TBPs. Unlike Trip1 and Sug1, the TBPs from these divergent species are not functionally

FIG. 3 **a**, *In vitro* interaction of Trip1 and Sug1 with transcriptional activators. Bacterially produced GST-Trip1, GST-Sug1 or GST alone were bound to a glutathione-agarose column and incubated with equivalent amounts of the indicated 35 S-labelled TR produced by *in vitro* translation. Specifically bound proteins, corresponding to ~5–10% of the input, were released by glutathione and resolved by SDS-PAGE. GST-Trip1 also bound RXR specifically, but did not bind luciferase, glucocorticoid receptor or several other control proteins. In other experiments, GST-Trip1 and GST-Sug1 showed a more equivalent binding of TR. **b**, Interaction of Trip1 and Sug1 with the transcriptional activation domains of GAL4 and VP16. Sug1, Trip1 and MSS1 were labelled by *in vitro* translation and incubated with either GST alone or with the activation domains of GAL4 (GST-34) and VP16 (GST-VP16) bound to a glutathione-agarose column. Proteins eluted by glutathione were resolved by SDS-PAGE. The size difference between the Trip1 and Sug1 proteins is a consequence of the presence of 6 × His and Myc epitope tags on the former.

METHODS. For production of GST fusion proteins, derivatives of the pGEX series of expression vectors containing either full-length Trip1 and Sug1 sequences or fragments consisting of the transcriptional activation domains of GAL4 or VP16 (ref. 7) were generated. Proteins were produced in *E. coli* as described¹⁹. For expression by *in vitro* translation, coding regions were inserted into the T7HXP/myc vector, a modification



of the pT7flu vector²⁰ which includes both 6 × His and Myc epitope tags. *In vitro* translation was carried out using the TNT-coupled transcription translation system (Promega). For protein interactions, GST proteins were bound to glutathione-Sepharose-4B beads (Pharmacia) in binding buffer (50 mM potassium phosphate pH 6.0, 100 mM KCl, 10 mM MgCl₂, 10% glycerol, 1% Tween20). Beads were washed once with binding buffer and incubated for 60 min at 4 °C in the same buffer containing 10 mg ml⁻¹ *E. coli* extract to block nonspecific binding, with equivalent amounts of the various proteins labelled with 35 S-methionine by *in vitro* translation. Unbound proteins were removed by three washes with binding buffer and specifically bound proteins were eluted with 50 mM reduced glutathione in 50 mM Tris, pH 8.0.

interchangeable^{17,18}. The ubiquitous expression of Tripl1 is also consistent with its postulated transcriptional role in mammalian cells. Although this expression substantially complicates functional analysis, a specific interaction with the receptors in mammalian cells is confirmed by the observation that Tripl1 overexpression specifically inhibits transactivation by both TR and RXR in appropriate cotransfections (not shown). Such a negative effect would be expected for overexpression of an individual component of a multiprotein complex such as the mediator¹¹, particularly one responsible for interaction with a specific target. We conclude that the identification of Tripl1 as a potential mediator of activation by Tr and other transcription factors is an important extension of the cellular roles of CAD proteins, raising questions regarding the role of these ATPases in transcriptional activation. □

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ERRATUM

Structural determinants of peptide-binding orientation and of sequence specificity in SH3 domains

Wendell A. Lim, Frederic M. Richards & Robert O. Fox

Nature **372**, 375–379 (1994)

IN Table 1 of this letter, binding positions that absolutely require proline are enclosed in a bold box; for the minus class of binding sequences, the P₂ binding positions should be boxed, and not the P₁ positions indicated in the table as published. □

CORRECTIONS

Detection of a γ-ray burst of very long duration and very high energy

K. Hurley, B. L. Dingus, R. Mukherjee, P. Sreekumar, C. Kouveliotou, C. Meegan, G. J. Fishman, D. Band, L. Ford, D. Bertsch, T. Cline, C. Fichtel, R. Hartman, S. Hunter, D. J. Thompson, G. Kanbach, H. Mayer-Hasselwander, C. von Montigny, M. Sommer, Y. Lin, P. Nolan, P. Michelson, D. Kniffen, J. Mattox, E. Schneid, M. Boer & M. Niel

Nature **372**, 652–654 (1994)

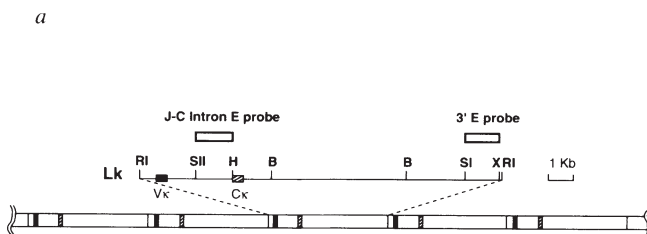
THERE is a typographical error in one of the superscript numbers in the caption to Fig. 3, inset. The second sentence should read “The best fit to the 30–30,000 MeV spark-chamber data is $(1.3 \pm 0.4) \times 10^{-7} (E/86 \text{ MeV})^{-(2.83 \pm 0.64)}$ photons cm⁻² s⁻¹ MeV⁻¹.” □

Affinity maturation leads to differential expression of multiple copies of a κ light-chain transgene

Francisco Lozano, Cristina Rada, John M. Jarvis & César Milstein

Nature **363**, 271–273 (1993)

WE have discovered an error in Fig. 2a of this Letter. The correct figure is shown here. This correction does not affect the arguments or conclusions presented in our paper. □



Nature's sister journals in March

As a service to those readers of *Nature* who do not see its sister journals regularly, the following pages refer to some of the content of *Nature Genetics*, *Nature Structural Biology* and *Nature Medicine*.

Continuing hunt for diabetes genes targets unique mini-satellites

THE early-onset form of diabetes appears to be associated with a tandemly repetitive DNA sequence of variable overall length upstream of the insulin gene on human chromosome 11 (11p15.5). That conclusion, of interest in its own right, is reminiscent of how variable numbers of repetitive elements, usually, are associated with various inheritable neurological diseases, Huntington's disease, for example.

The role of the repeating elements in the genetics of early-onset diabetes (also known as "type I" and "insulin-dependent" diabetes, or "IDS") is outlined by two articles in the March issue of *Nature Genetics*. The new developments are the outcome of a decade-long search for the inheritable correlates of early-onset diabetes. No fewer than six genetic determinants of IDS have now been identified, of which the principal is a locus in the LA complex on chromosome 6.

Linkage studies have previously located a susceptibility locus (called) to a 4.1 KB region of chromosome 11 that includes the insulin gene. A large group from the University of Oxford, including S. T. Benett and J. A. Todd, together with collaborators from Norway, Denmark, Birmingham (England) and France, have now been able to show that coincides with the Variable Number Tandem Repeat (*VENTURE*) know for a decade to lie upstream of the start-point of the insulin gene (S. T. Benett *et al.* *Nature Genet.* **9**, 284-292; 1995).

As mini-satellites go, this *VENTURE* is unusual in that the repeating element is 14 (sometimes 15) nucleotides long. A total of at least 11 variants of the sequence have been recognised, all of which are rich in guanine. Haploid chromosome sets may differ from each other in the number of repetitive units (from fewer than 40 to more than 150) and in the occurrence and order of variants of the basic repetitive sequence.

The proof that *VNTR* alleles determine susceptibility to IDDM rests on studies of parental transmission to offspring (diabetic or otherwise) in 425 families from Britain, France and the United States, which has in turn been made possible by a successful technique for PCR fluores-

cence-linked amplification of microsatellites, followed by electrophoresis with acrylamide and urea gel to give an estimate of the size of the *VNTR* allele.

One finding is that the shorter alleles are associated with susceptibility to early-onset diabetes, but there is one ironical exception. The allele whose length is 698 base-pairs ($14 \times 50 = 700$) appears to be protective; the irony is that this was the version of the mini-satellite first sequenced. The authors also conclude that most short alleles (but not that numbered 698) are associated with enhanced insulin expression. There is also evidence of maternal and paternal imprinting (differences of the efficacy of the differently sourced genes) in the diploid genome.

In an accompanying paper, Guilia Catignani Kennedy, Michael S. German and William J. Rutter from the University of California, San Francisco, show that the *VNTR* locus upstream from the insulin gene is capable of binding to molecules of the transcription factor known as Pur-1 (*Nature Genet.* **9**, 293-298; 1995). Their objective is to study the effects of repetitive units of different sequence on the efficacy of gene promoter regions in which they are included, using a novel *in vitro* system in which pancreatic β -cells are used to assess the effects of various *VNTR*

sequences within the promoter region on the expression of reporter genes. A complication for the further study of susceptibility to IDDM is the finding that there are, indeed, significant differences between the effects of different nucleotide sequences.

Kennedy *et al.* that some of their conclusions may be undermined by the finding of Bennett *et al.* that the "short" allele used in their study may be that found to counteract susceptibility to diabetes. They go on to make two other important points:

First, the location of the *VNTR* locus within the promoter region of the insulin gene does not, of itself, account for the aetiology of IDDM, which involves the auto-immune destruction of pancreatic β -cells, but the excess of insulin production above some threshold may be the trigger for such a reaction.

Second, the *VNTR* locus is found, among vertebrates, only in primates, suggesting a relatively recent evolutionary origin for this mini-satellite and even raising questions about the function of this nucleotide complex other than to confer susceptibility to IDDM.

The same issue of *Nature Genetics* includes a report by a largely French group (based at two INSERM units at Paris) of a link between a point-mutation of the human glucagon receptor gene and the occurrence of "non-insulin dependent" diabetes (or NIDDM). The glucagon receptor is a transmembrane protein whose gene occupies more than 5.5 kb on human chromosome 17, up against the "q" telomere, and is coupled cytoplasmically to G-protein.

J. Hager *et al.* (*Nature Genet.* **9**, 299-304; 1995) describe a significantly greater incidence of this mutation among patients with NIDDM from France and Sardinia (where the overall incidence of NIDDM exceeds 10 per cent for those over 60). They also show that the affinity of glucagon for the mutated receptor is much reduced, compared with binding to the unmutated version.

Even so, the mutated glucagon receptor is not an unequivocal indicator of the occurrence of NIDDM; in the Sardinian population, this version of the gene is only three times more common in late-onset diabetics than in the general population. But, intriguingly, the effect of the mutation is to make the amino acid sequence of a highly conserved region of the glucagon receptor identical with that of the rat. □

**nature
genetics**

Also in *Nature Genetics*, March:

■ The mouse gene *Mash2*, which encodes a transcription factor required for trophoblast development, is genomically imprinted (in favour of the maternal gene) and is located within a cluster of such genes on mouse chromosome 7.

■ Foreign genes may be transferred to mouse embryos at any stage of gestation by injecting plasmid-lipopolyamine complexes into pregnant females.

■ A point mutation of the gene for the copper-transporting protein ceruloplasmin has been demonstrated to be responsible for inherited systemic haemosiderosis, but it is not clear why neurological and other organ damage is caused by the deposition of iron, rather than copper, in affected tissues.

■ Imprinting of the *Xist* gene (on the X chromosome) appears to be linked with methylation.

'Catalytic triad' modified

A NEW structure of an esterase from the potato scab-inducing bacterium *Streptomyces scabies* (Y. Wei, *et al. Nature struct. Biol.* 2, 218–223) may compel a reassessment of how the so-called "catalytic triad" functions in enzymes generally. The 2.1 Å structure has been obtained by Z. S. Derewanda's group at the University of Alberta with collaboration from the University of Minnesota and the Novo-Nordisk Research Laboratories at Copenhagen.

A detailed structure of the chymotrypsin molecule a quarter of a century ago first drew attention to the involvement of three amino acids, from different parts of the molecular sequence, in the active site of the enzyme. Since then, the same catalytic triad, with variations, has been found in a range of enzymes all concerned with the breakdown of biological material.

There is often no other homology than three amino acids, a serine, a histidine and an acidic residue (usually either glycine or asparagine), brought together from different points in the protein sequence. The serine is the chemically reactive group, making a nucleophilic attack on the enzyme's substrate. In chymotrypsin the chemical reactivity was thought to be achieved by a 'proton-shunt' in which the histidine withdraws a hydrogen atom from the serine residue and itself loses a proton to the acidic aspartate. □

Proteasome function revealed

ALTHOUGH there are many forms of the catalytic triad, it has hitherto been thought that the third acidic member is essential to stabilize the positively charged histidine. Now, in the esterase from *S. scabies*, a bulky aromatic tryptophan that cannot function as an acid turns out to be the third member of the triad. An analogous situation has been found in a catalytic antibody, in which a serine and a histidine appear to function as if in a catalytic triad without the equivalent of a third acidic member.

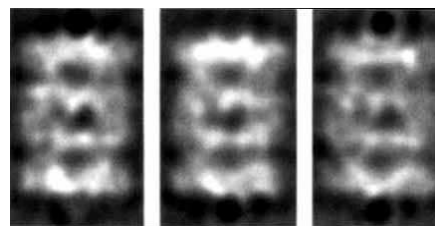
In the light of the new *S. scabies* esterase structure, the authors suggest that the role of the third member of the catalytic triad may not be electrochemical, but rather that of correctly orienting the histidine, in which case the term 'catalytic diad' may be more appropriate.

An elegant series of experiments has now thrown light on the way in which the cytoplasmic structures called proteasomes differentiate between proteins to be degraded by proteasomes and those left intact. (T. Wenzel & W. Baumeister *Nature struct. Biol.* 2, 199–204; 1995).

Proteasomes are cylindrical assemblages of twenty-eight proteins arranged as a stack of four seven-membered rings, together with a number of accessory proteins. They are present in both eukaryotes and prokaryotes, being responsible for the destruction of damaged and misfolded proteins as well as short-lived regulatory proteins marked for destruction by tagging with the protein ubiquitin.

Wenzel and Baumeister, from the Max-Planck institute at Martinsried (Munich), have used proteasomes from the archaeobacterium *Thermoplasma acidophilum* to show that only fully unfolded proteins are degraded. They suggest that degradation takes place within the proteasome and that only proteins which can thread into its inner cavity are degraded.

Graphic evidence for that is provided by the observation that when a bulky gold particle was attached to the B-chain of



insulin, which is normally swiftly degraded, the peptide became resistant to degradation. Indeed, such a gold particle can be seen under the electron microscopy plugging the ends of the proteasomes (as in the figure).

This does not explain how the tagging of proteins with ubiquitin leads to their destruction, but the authors suggest that the accessory proteins at the ends of the proteasome, which were not present in this study, may act as 'reverse-chaperones' selectively unfolding proteins so that they can be fed into the cellular equivalent of a shredding machine. □

Glycosylation of IgG and synovial inflammation in rheumatoid arthritis

THE possibility that the activation of complement in the causation of inflammation in rheumatoid arthritis is brought about by abnormal glycosylation of IgG molecules is suggested by Rajneesh Mulhotra *et al.* and Raymond A. Dwek, respectively from the MRC Immunochemistry Unit and the Glycobiology Institute of the Biochemistry Department, University Oxford, in the March issue of *Nature Medicine*.

With collaborators, the two groups build on earlier observations that the glycosylation pattern at the Asn 297 site of IgG is abnormal in rheumatoid arthritis, with the loss of either one or two of the terminal galactose residues in a normally branched chain (R. Mulhotra *et al. Nature Med.* 1, 237–243; 1995). The authors now demonstrate that, in the absence of the

terminal sugars, binding to mannose binding protein (MBP) is enhanced and that one consequence is the activation of complement.

This chain of events is substantiated both biochemically and by structural studies, which reveal a similarity of the structure of multimers of MBP with the structure of C1q, the presumed initiator of the complement cascade, both of which include regions resembling collagen in structure.

The inference is that the presence of IgG antibodies lacking terminal galactose residues may cause the chronic inflammation of the synovium characteristic of rheumatoid arthritis, but the authors raise the question whether imperfectly glycosylated IgG may have an earlier function in the causation of the disease. □

nature medicine

Nature Medicine — other points

- A correspondent from the Queen's University, Belfast, pleads that optimism about the use of gene therapy for the treatment of cystic fibrosis (CF) should not conceal from physicians and their patients that CF is a systemic disease, affecting other organs than the lungs, and that there is a "need to help patients live with the reality of their disease".
- The observation, during the use of adenovirus vectors for the delivery of human CFTR genes to bronchial tissue, of inflammation linked with the release of interleukin-6 has led to the further observation that this cytokine is also produced in the inflammatory

lesions in the CF lung.

- On the assumption that the failure of grafts of fetal tissue in the treatment of rat models of Parkinson's disease may be a consequence of oxygen toxicity, transgenic cells from the substantia nigra designed to over-express (Cu-Zn)-superoxide dismutase are reported to survive for longer.

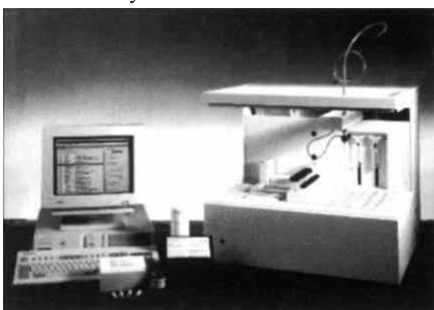
- The finding in a small number of people with acute depression of antigens belonging to the Borna virus, originally recognized as responsible for neurological disease in horses, suggests a new line of enquiry for psychiatrists with an interest in PCR techniques.

Singling out and setting apart

Showing true colours, this week's segment features plasmid purification kits, a two-dimensional vertical DNA electrophoresis system, a particle separator, an HPLC system and a low-cost protein purification system.

AN anion-exchange resin packed into disposable columns forms the basis of a new range of **plasmid purification kits** now available from Hybaid (*Reader Service No. 100*). When used with a modified alkaline/SDS lysis procedure, the Jetstar kits enable high-purity plasmid DNA to be obtained in stated times of less than one hour. Three kits offer options for the size of plasmid preparation: the mini kit can yield up to 20 µg of DNA; the midi 100 µg; and the maxi 500 µg. Further flexibility is provided with an option to prepare DNA with or without RNase; the latter being particularly important if a subsequent *in vitro* transcription experiment is to be performed. The yield of plasmid DNA is said to be between 80–90 per cent with the quality of DNA being higher than that obtained using double CsCl gradients. Each kit is supplied with all the necessary components, solutions and protocols. In addition, as the columns operate under gravity flow, there is no need for any additional instrumentation.

Vistra DNA Systems now offers the Vistra DNA Labstation 625 for automated **DNA sequencing template and reaction preparation** (*Reader Service No. 101*). Unattended, the labstation can prepare up to 72 gel-ready sequencing samples per day, for manual or automated sequencing. It produces purified DNA templates starting with either bacterial cultures, phage supernatant or PCR products. Amersham FMP magnetic separation technology enables the labstation to perform these steps without centrifugation, chromatography or other intervention. The system supports both Sequenase and Δ Taq procedures for radiolabelling, single dye/four lane, or four dye/single lane reactions. For Δ Taq sequencing, built-in thermal cycler and oil overlay systems are activated according to the protocol selected. A cooling block preserves the activity of heat-labile enzymes, and separate disposal systems prevent the production of costly mixed hazardous waste.



The Vistra DNA Labstation.

Waters has released the Millennium 2.1 **chromatography manager** (*Reader Service No. 102*). Included in this package is QuickStart, a module that makes running HPLC methods easier by prompting users through the method development process in a step-by-step fashion, from initial injection to the reporting of results. Enhanced photodiode-array software is designed to allow the automated extraction of multiple wavelengths of chromatographic data. The data can be viewed in three-dimensional review or processed automatically using mathematical algorithms. Also available is an option for polymer characterization which provides a combination of differential-refractive index and viscometry-detection capabilities to determine the molecular weight and construction of sample compounds. For control of the company's Quanta 4000E capillary electrophoresis or capillary ion analyser, an optional control package incorporates reference peaks, time connected area and isomigration mode to ensure reliable peak identification and quantitation.

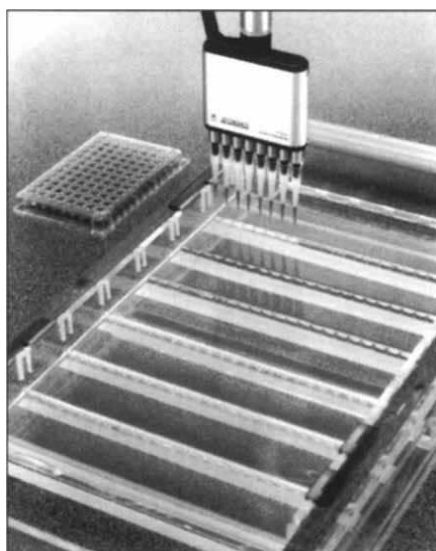
■ Electrophoresis equipment

Alpha Laboratories has introduced a new reagent for enhanced **agarose gel electrophoresis** (*Reader Service No. 103*). Infinity agarose enhancer, a modified polysaccharide, is designed to improve the performance of agarose with respect to resolution, gel transparency and gel strength. It is used in combination with the agarose of choice to create an enhanced gel matrix. These gels can be prepared as a general-purpose agarose (<50 kb), high-resolution agarose (>1,000 bp) or pulsed field agarose (>50 kb) by varying the agarose concentration and type of agarose. Infinity retains the solidification and remelting characteristics of the agarose with which it is used. The manufacturer states that these gel additives are guaranteed to be DNase and RNase free and have been thoroughly performance tested in applications such as restriction enzyme digests, PCR and random priming.

The Ingeny PhorU-2 is a **vertical electrophoresis apparatus** is designed for two-dimensional DNA electrophoresis and can hold two lanes to facilitate spot pattern comparison of two different samples (*Reader Service No. 104*). The unit also lends itself to large-scale denaturing gradient gel electrophoresis analysis where as many as 40 samples can be run simultaneously. A U-shaped spacer facilitates leak-free gel casting without the need for plugs

or rubber seals. The unit also comes with built-in electrodes, a 40-well comb, one set of glass plates and safety power leads.

The latest addition to Anachem's range of **horizontal electrophoresis units** is the H5 set — designed to allow loading of up to 138 samples on one gel (*Reader Service No. 105*). The 30 cm × 20 cm unit is designed to accept six combs of 23 samples each. This enables those using 96-well PCR



Anachem's H5 electrophoresis set.

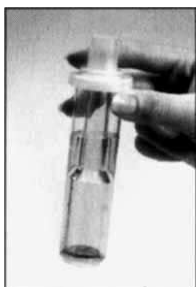
machines with an 8 × 12 matrix to screen all their samples on one unit. The H5 unit has an acrylic construction; a removable ultraviolet-transparent gel tray; user-replaceable platinum electrodes; and recessed, power connectors that are an integral part of the lid. Gels can be cast directly in the unit.

Owl Scientific has released the Puffin single-sided, **mini-vertical electrophoresis system** (*Reader Service No. 106*). This system includes an upper buffer chamber that extends the length of the gel providing even heat distribution by bringing the buffer into direct contact with the gel. It has spring loaded, full length side clamps to provide leak-free clamping. An optional, built-in cooling chamber is available that is designed to transfer heat away from the gel for fast runs and flat, even banding patterns. Platinum electrodes are resistant to corrosion and have increased surface area for improved field uniformity. The system is intended for SDS-PAGE, gradient gels, agarose gels and SSCP detection. The

Puffin 10 cm × 10 cm mini-gel system comes complete with one blank glass plate, one notched glass plate, one alumina notched plate, one spacer set and two combs. The system will accept most pre-cast gels.

■ Centrifugation

Amicon's new Centriprep **centrifugal particle separator** is designed for removing debris such as cells, beads or other particles from biological samples (*Reader Service No. 107*). During centrifugation, the device minimizes the accumulation of particles on the membrane surface which would otherwise block the filter and diminish its effectiveness. Centrifugal force drives suspended particles away from the filter surface while the pressure resulting from centrifugation forces membrane-permeable material through the membrane. The device has a membrane porosity of 0.2 µm and is capable of purifying up to 15 ml.



Particle separator from Amicon.

Hettich now offers the Mikro 24-28 **microcentrifuges** (*Reader Service No. 108*). These models are designed for high speed, high RCF (23,000g). They provide a large capacity, short run-up and slow-down times and quiet operation. A choice of angled rotors (12 1.5 ml and 2.2 ml and 48 1.5 ml to 2.2 ml); a drum rotor (60 1.5 ml to 2.2 ml) and a swing-out rotor (24 1.5 ml to 2.2 ml) cater to a wide variety of applications. A rotor detection system automatically sets the maximum speed of each rotor. Speed radius, RCF, running time, accelerating level, braking level, as well as temperature in the refrigerated models can be entered through the foil keypad. The refrigerated model can be controlled from -10 °C to 40 °C.

Centrifugal **elutriation** from Beckman Instruments is designed to separate large quantities of cells for biochemical purposes (*Reader Service No. 109*). Also known as counterflow centrifugation, this technique is said to provide a gentle, high-yield method for isolating cells for immunology, tumour biology and many other areas where cells need to be harvested. This method does not require density gradient chemicals, does not produce a pellet of cells and does not require high g-forces. Large cell populations can be recovered, as many as 10¹⁰ cells of a specific size, in stated times that are often less than one hour. Cells from 2 µm to

50 µm can be separated as long as they have a 1:1.2 diameter ratio. The Beckman elutriation system consists of a rotor assembly, a separation chamber, a strobe assembly and a flow system. A Beckman J2 or J6 series centrifuge can be used, but must be equipped with a viewing port in the centrifuge door. A dedicated instrument is not required; elutriation components can be removed for other centrifuge applications.

A new **centrifuge** from DuPont can help researchers complete experiments faster by offering automated operation at a top speed of 26,000 r.p.m. (*Reader Service No. 110*). The Sorvall RC-26 plus is fully programmable and micro-processor controlled. Run parameters can be entered in advance with the control-options mode, and set-up is completed in three simple stages. Other design features include a maximum RCF of 70,450g, 3-litre capacity, a reduced noise level and ozone-friendly refrigeration. This machine is designed for fast acceleration, shorter run times and quiet operation by utilizing a high torque, brushless direct current drive with Sorvall lightweight, fixed-angle aluminium rotors. The rotors are said to weigh up to 50 per cent less than conventional models.

Savant has released three new digital **microcentrifuges** in the µSpeedFuge line (*Reader Service No. 111*). There is the air-cooled SFA13K; the high-capacity, air-cooled SFA13K plus; and the refrigerated SFR13K. This line is designed for quiet operation. Digital programming can set values for RCF up to 13,000g and run times of up to 60 min. These models are said to have rapid acceleration and deceleration with pulse function and elapsed time shown on a large LED display. Rotors are constructed of autoclavable, corrosion-resistant polypropylene.

Life Sciences offers the IEC Centra GP8 **benchtop centrifuge** (*Reader Service No. 112*). This unit has a 3-litre capacity and offers a rotor adapter system for tubes, blood bags, bottles (0.25 ml to 750 ml) and microplates. A 35-programme memory with locking feature is designed to ensure reproducible results and eliminate tampering. Features for operator safety include sealed buckets and rotors, a cover interlock system and imbalance protection with automatic shut-off. For temperature sensitive applications the GP8 refrigerated model has a rapid heating and cooling programme and temperature alarm.

■ Chromatographs

Chrompack offers injection versatility with the CP-9001 **gas chromatograph** (*Reader Service No. 113*). This model fea-

tures the CP-9110 temperature programmable on-column injector for direct programming of popular injection techniques, such as packed, split/splitless, wide bore and on-column injection. When used with automated samplers, the CP-9001 matches sample temperatures to the on-column injector. This is said to be suitable for environmental applications where volatile samples must be analysed with high precision. When combined with the company's Ultimet columns, this system can also handle high-temperature analyses (up to 450 °C).

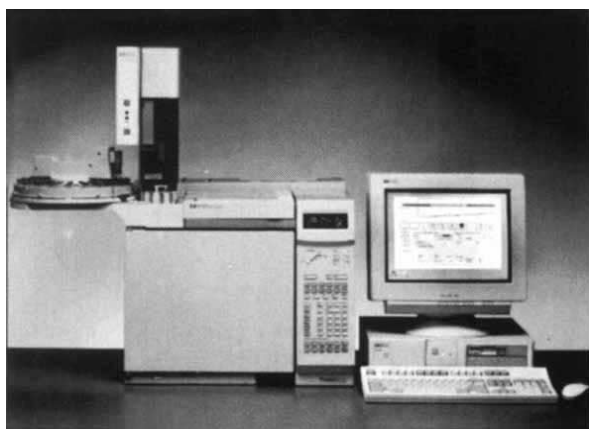
Hitachi has recently introduced the ConcertChrom series **HPLC system** (*Reader Service No. 114*). This series includes an isocratic and a quaternary pump system; diode-array ultraviolet, visual and fluorescence detectors; autosamplers; an integrator; and PC-based data system. Each of the systems modules



Hitachi ConcertChrom.

is designed to be stacked in a compact, 26-cm wide tower configuration. The manufacturer states that it can accommodate previous Hitachi HPLC instruments without any special add-ons. The ConcertChrom system focuses on the reporting of good laboratory practice. It runs on a Windows NT operating system, has a security logbook and networking capabilities.

Hewlett Packard has introduced the HP 6890 series integrated **gas chromatography system** (*Reader Service No. 115*). This system features electronic, pneumatic controls that regulate all gas pressures and flows. On-board sensors automatically compensate for changes in ambient temperature and barometric pressure differences to achieve more accurate reproducible results. This system is designed to reduce operating costs through gas savings, faster set-up times and reduced equilibration time. The HP 6890 series pneumatics optimize performance of the split/splitless inlet. Other features include direct access control keys, a five-method storage capability and HP-IB and RS-232 communication ports that provide easy system interconnection. The automated features are designed to increase laboratory produc-



Hewlett Packard's 6890 gas chromatography system.

tivity: clock-time programming loads and runs a method for unattended operation, pre-run programming enters conditions and prepares the system for the next injection, the gas saver function reduces the split flow rate after the start of a run and between runs to conserve expensive carrier gas, and post-run programming starts after the chromatographic run to reduce the amount of system resources wasted.

■ Columns and media

Phenomenex has developed a series of non-aqueous GPC columns designed for **high-temperature analysis of polymers** (*Reader Service No. 116*). Based on highly crosslinked poly(styrene-divinylbenzene), the Phenogel 5- μm and 10- μm materials are designed for mechanical, thermal and chemical stability. The intended result is a wider solvent compatibility and resistance to gel shrinking and swelling during solvent switching and prolonged operational life at higher temperatures ($>150^\circ\text{C}$). It is also meant to be resistant to most 'aggressive' organic solvents and is said to tolerate frequent mobile phase changes. Amine and acid modification of the mobile phase, with up to one per cent water, is also said to work without any difficulties.

Pharmacia Biotech offers Source media for **hydrophobic interaction chromatography** (HIC) of proteins and peptides (*Reader Service No. 117*). Source 15ETH (ethyl), 15ISO (isopropyl) and 15PHE (phenyl) have been developed with an emphasis on reproducibility and scalability and are said to be useful for the final stages of large-scale purification where trace contaminants and close variants need to be removed. These media have been developed to maintain resolution at high flow rates and sample loads. Source HIC media can be packed in standard and high-performance laboratory-scale columns and run on an FPLC, HPLC or BioPilot system with separation times of 2 min to 1 h.

Rockland Technologies has introduced two new HPLC columns for **resolving proteins and peptides** (*Reader Service No. 118*). Zorbax 300SB-C3 and 300SB-CN are wide-pore (30 nm), reversed-phase columns produced with monofunctional silanes that use bulky side chain groups to sterically protect the siloxane bond against hydrolysis. The resulting columns are stated to offer superior stability for short-chain, reversed-phase col-

umn packings under acidic conditions, even at elevated temperatures.

Protec-RP **base-deactivated columns**, from ES Industries are designed to eliminate the problem of peak tailing (*Reader Service No. 119*). These columns are designed to enable the analysis of basic compounds without the use of amine-modified mobile phases. Improved peak shapes are said routinely to be obtained under reversed-phase conditions for strongly basic and polar organic compounds, as well as pharmaceuticals from natural or synthetic sources. The manufacturer states that these columns meet compliance requirements for lower quantitation levels during the development of new drug assays. Trace levels will elute with minimal peak tailing using simple buffers containing mobile phases at low ionic strength. To assist with method development, ES Industries offers column pretesting services to ensure system suitability.

Toso Haas TSK-GEL NPR columns are designed for fast, high-resolution **analyses of proteins, peptides and polynucleotides** (*Reader Service No. 120*). The columns are packed with 2.5 μm polymeric nonporous beads which force the sample to move around them instead of through pores. This is intended to enhance speed and resolution. These columns are said to provide high sample recoveries and low solvent consumption. TSK-GEL NPR columns are designed for applications such as methods development, quality control, process monitoring and basic research.

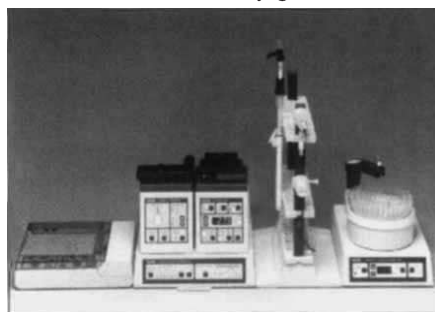
Shandon Hypercarb **PCB columns** have been designed for environmental analyses involving pesticides, PCBs, PCDDs and PCDFs (*Reader Service No. 121*). Hypercarb columns require only hexane as a solvent and are said to make analysis quicker and less complicated than more complex solvent methods. These columns can be used to determine the concentration of pollutants in soil samples and

can distinguish the less toxic PCBs from more toxic, non-ortho chlorine substituted PCBs.

■ Misfits

Gilson Medical Electronics offers a new brochure that explains how to prepare and **process raw biological samples for HPLC** with the Asted XL sample analysis system (*Reader Service No. 122*). Suitable for both routine analysis and method development, Asted XL removes the macromolecular compounds present in a sample matrix: proteins, polysaccharides and glycolipids. It enriches analytes on a trace enrichment cartridge and injects them onto the HPLC column. A series of chromatograms show the efficiency of the sample clean-up and the wide range of applications.

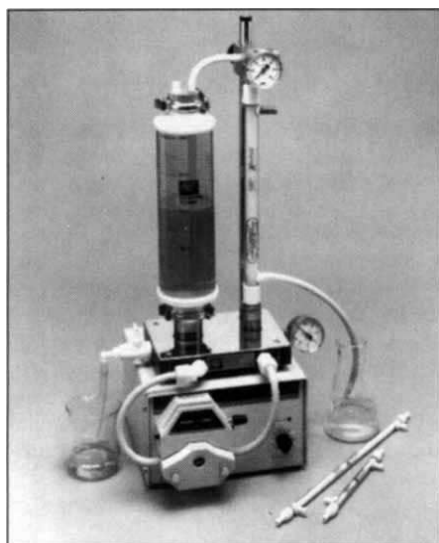
Bio-Rad has designed the Econo System for **low-pressure protein purification** (*Reader Service No. 123*). The Econo System is designed to be a low-cost chromatography workstation. It enables the user to form a binary gradient, collect



The Bio-Rad Econo System for proteins.

protein peaks by time windows or peak threshold and walk away from the lab bench while separation is in progress. The system includes a peristaltic pump, ultraviolet monitor with 280/254 nm detection capabilities, an adjustable rack, a controller, a fraction collector, as well as other accessories needed for protein separation. Icon-driven programming makes the system easy to use; it can also be upgraded for complete automation, from sample injection to fraction collection.

The QuixStand benchtop system, now offered by A/G Technology, is designed for **concentration and/or diafiltration of biological solutions** (*Reader Service No. 124*). This laboratory-scale, cross-flow, hollow-fibre system is capable of processing volumes of up to 5 litres. The system includes a variable cartridge support stand, inlet and outlet pressure gauges and 400-ml and 1-litre reservoirs, which can be pressurized for gentle recirculation of labile solutions. It is self-contained and incorporates a precision control backpressure valve and a process pump with a variable speed drive and a nominal maximum recirculation rate of 2 l min⁻¹. The low hold-up volume



QuixStand from A/G Technology.

design is intended to allow concentration to as low as 25 ml. The QuixStand system accommodates the company's Xampler hollow-fibre ultrafiltration cartridges ranging from 3,000 to 500,000 nominal molecular weight cutoff and microfiltration cartridges in pore sizes from 0.1 μm to 0.65 μm . Membrane areas range from 24 cm^2 to 1,070 cm^2 .

Packard Instruments has introduced a new line of ultra-high purity (UHP) **zero air generators** (Reader Service No. 125). These models are designed as alternatives to high-pressure gas cylinders. They provide a continuous flow of hydrocarbon-free, ultra-pure, zero-grade air from an existing compressed air supply. These models produce up to 2.5 l min^{-1} of zero air at pressures of 15 Pa to 905 Pa on demand. The UHP zero air generators reduce the total hydrocarbon content to less than 0.1 p.p.m., measured as methane. They are intended to eliminate the expense and potential hazards of high-pressure gas cylinders.

For high-resolution **HPLC analysis of oligosaccharides**, Oxford GlycoSystems offers the Signal labelling kit and the GlycoSep HPLC column set (Reader Service No. 126). The Signal labelling kit uses 2-aminobenzamide (2-AB) to label oligosaccharides at their reducing terminus. The manufacturer states that from as little as 25 picomoles to 50 nanomoles of sample oligosaccharide labelled glycans can be prepared in high yield (>85 per cent) in up to 2 h. The labelled oligosaccharides can then be analysed by two-dimensional HPLC: a charge-based separation on GlycoSep C and a hydrophobicity-based separation on GlycoSep H. Used together, these columns can separate oligosaccharide mixtures released from glycoproteins. The GlycoSep HPLC column set contains both columns together with standards and pro-

ocols for the separation of oligosaccharide mixture. A standard HPLC system with a fluorescence detector is required.

Lazar Research Laboratories introduces a gauge for the accurate **tightening of HPLC fittings** (Reader Service No. 127). Variations in torque can lead to overtightening or undertightening, resulting in leaks or damage to fittings or expensive HPLC components. The Acutorque tightening system accurately measures and displays the amount of torque being applied to the fitting as it is being tightened. A specially designed alligator-jaw gripping device attaches onto knurled type finger-tight fittings while a specially designed socket fits directly onto hexagonal metal fittings. These are designed not to interfere with HPLC tubing protruding from the fitting. Acutorque can tighten fittings manufactured by Waters, Upchurch, Swagelok, SSI, Valco, Parker, Rainin and Alltech.

Ultra Violet Products has introduced a new **thin-layer chromatography-plate documentation and analysis system** — the Chromato-Doc 7500 (Reader Service No. 128). Analytical capabilities are provided by the Chromato-Pro software package, which is available for both IBM PC-compatible and Apple Macintosh computers. This system is designed to view, document, analyse, print and archive TLC plate images in a multitude of laboratory environments. The manufacturer states that image costs are low. The Chromato-Doc 7500 system is comprised of a black and white, high-resolution charge-coupled device camera with zoom facility; a stand; an ImageStore 7500; a colour monitor; and a thermal printer. Once the image has been captured it can be enhanced or annotated before being saved to a floppy disk for transfer to a suitably located computer — numerous file outputs are available. Hard-copy output is via the thermal printer. Other options include a cabinet, a bench-top darkroom with built-in ultra-violet and white light illumination.

■ Catalogues and brochures

The Gelman Sciences' **biotechnology-microfiltration products catalogue** contains products for cell culture, molecular biology, microbiology, analytical sample preparation, routine and critical laboratory applications (Reader Service No. 129). These products include disc filters, blotting and affinity membranes, syringe filters, medium volume filters, microbiological media and accessories, vent and solvent filters, capsules, minicartridges and filter holders and funnels. The 96-page catalogue contains colour-coded product categories to assist in selection.

The 1994–1995 Jones Chromatography **catalogue** contains 354 pages of high quality equipment and accessories for LC, HPLC, GC and SPE (Reader Service No. 130). New product lines released in this catalogue include Isolute SPE columns, Xactflow pipettes, the model 7955 column heater/cooler and the JCL6000 Windows chromatography data system.

These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.

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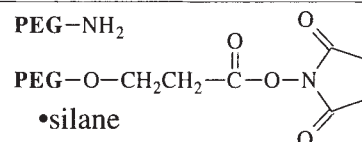
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